

VOLUME 67 • MAY 1959 • NUMBER 5

# PATHOLOGY

A Periodical Devoted to General and Experimental Pathology

**Neoplasms and Other Diseases in Aging Rats  
Following Partial- and Total-Body X-Irradiation**

*Baldwin G. Lamson, Marta S. Billings,  
and Leslie R. Bennett*

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**Mechanism of Hematopoiesis**

**Hematopoietic Effects of Serum Albumin**  
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**The Architecture of the Conduction System in  
Congenital Heart Disease**

*Maurice Lev*

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VOLUME 67

MAY 1959

NUMBER 5

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Published monthly by the AMERICAN MEDICAL ASSOCIATION. Editorial and Circulation Offices: 535 North Dearborn Street, Chicago 10, Illinois. Publication Office: Thompson Lane, Box 539, Nashville 1, Tennessee.

Second-class postage paid in Nashville, Tennessee.

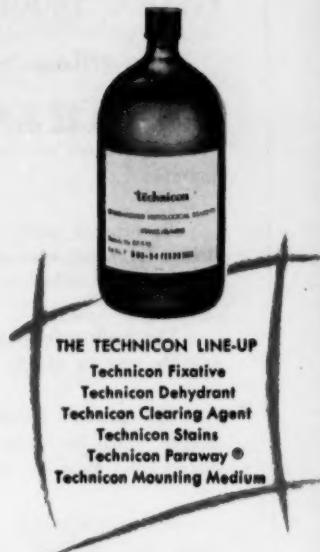
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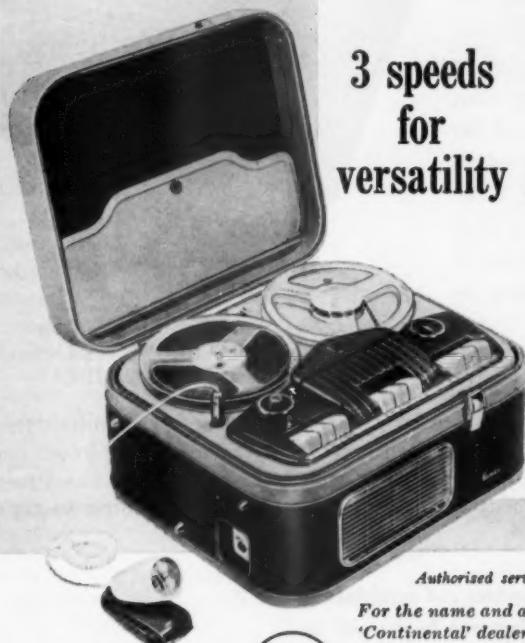
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## Neoplasms and Other Diseases in Aging Rats Following Partial- and Total-Body X-Irradiation

*Significance of Animal Data in the Evaluation of Somatic Radiation Hazards in Man*

BALDWIN G. LAMSON, M.D.; MARTA S. BILLINGS, M.D., and LESLIE R. BENNETT, M.D., Los Angeles

Adverse biological effects of radiation exposure may be broadly segregated into genetic and somatic hazards. The genetic hazard is related to radiation exposure to the gonads and will not be considered in the present discussion; the somatic effects of radiation become manifest in the exposed person himself and, therefore, present a more immediate problem than hereditary effects which will not become manifest in the present generation. Somatic effects include both acute and delayed consequences of radiation.

Acute radiation illness following within hours or days of total-body radiation exposure has been extensively studied. The acute radiation dose lethal to 50% of an exposed population within 30 days ( $L_D_{50/30}$ )

for a wide variety of mammals, ranging in size from mice and rats to dogs, swine, and probably man as well, falls within the range of 250 to 800 r when the radiation is expressed as midline absorbed dose (rad) in these species of varying size.<sup>1</sup> This similar acute response to radiation among many mammalian species is the basis for also extrapolating to man from laboratory animal data pertaining to *delayed* radiation effects, as will be brought out subsequently.

Our earliest understanding of delayed somatic radiation effects relates to the delayed changes in the tissues included within radiation fields either directed toward the treatment of neoplasms or inadvertently absorbed by early radiation workers. The characteristics of delayed radiation effects following local high-intensity exposure are now well known. After a long period, tissues in the radiation field become atrophic and fibrotic. Vessels become thick-walled or telangiectatic, and in extreme instances proliferative changes and malignancy, particularly of the skin in the irradiated area, may occasionally supervene. Much more rarely bone sarcoma arising in the irradiated area has been described. Histologic evaluation of tumors arising within these local irradiated areas have in some cases identified particular tumor types, such as spindle-cell variants of squamous carcinoma of the skin that are

Submitted for publication Aug. 28, 1958.

Read before the Section on Pathology and Physiology at the 107th Annual Meeting of the American Medical Association, San Francisco, June 24, 1958.

From the Departments of Pathology and Radiology and the Radiobiology Division of the Atomic Energy Project, School of Medicine, University of California at Los Angeles.

This paper is based on work performed under Contract No. AT-04-1 Gen. 12, between the Atomic Energy Commission and the University of California at Los Angeles, and in part under Contract No. AF 18(600)-1267, with the School of Aviation Medicine, United States Air Force, Randolph Air Force Base, Texas.

characteristic of radiation injury. The confinement of these late degenerative changes within the irradiated area convincingly relates these effects to the ionizing exposure. Late radiation changes of the type described are not usually extensively observed unless the radiation exposure exceeds 1,000 r. With modern fractionated radiation therapy these delayed tissue alterations are seldom a clinical problem following 6,000 r.

Severe delayed vessel alterations in association with atrophic tissue changes, so characteristic of local high-intensity radiation injury, are usually not observed in survivors of *total-body* or extensive partial-body irradiation, possibly for the simple reason that the approximate threshold dose for these tissue changes (1,000 r) is usually lethal when delivered to the major portion of the body. Obviously, the demonstrable delayed somatic effects following *total-body* irradiation must differ from the characteristic changes of x-ray injury that have become so well known from local high-level radiation exposures of several thousand roentgens.

Our knowledge of these delayed somatic changes following sublethal *total-body* irradiation, commonly referred to as the "late-effects syndrome," is based, of necessity, largely upon animal data. Evaluation of these delayed somatic radiation hazards has proven a slow and expensive problem. Animals must be observed throughout their life span for radiation injury, which may not become evident until many months or years after radiation exposure. Late effects of somatic irradiation may even occur after fractionated chronic exposures that are composed of individual radiation doses so small that acute radiation illness is never observed. We are concerned with a hidden silent damage that cannot readily be detected during its long latent period by usual clinical procedures. Furthermore, this latent period that precedes the development of manifest signs of late radiation injury may be a period of relatively good health, yielding a temporary false evaluation of the sequelae to be

later associated with the earlier radiation exposure.

Delayed radiation effects so far recognized have, like their acute counterparts, been remarkably similar in all mammalian species studied. The following discussion relates principally to the laboratory rat in order to allow use of illustrative data from our own laboratory bearing upon several aspects of this subject.

#### Late Effects Syndrome in Laboratory Rat

This late-effects syndrome following *total-body* radiation finally becomes manifest as shortened longevity, retardation of growth among growing animals, and an early onset of diseases that are usually prevalent in the later periods of the normal rat life span. This acceleration of time of appearance of diseases usually associated with old age has been referred to as premature or accelerated aging.

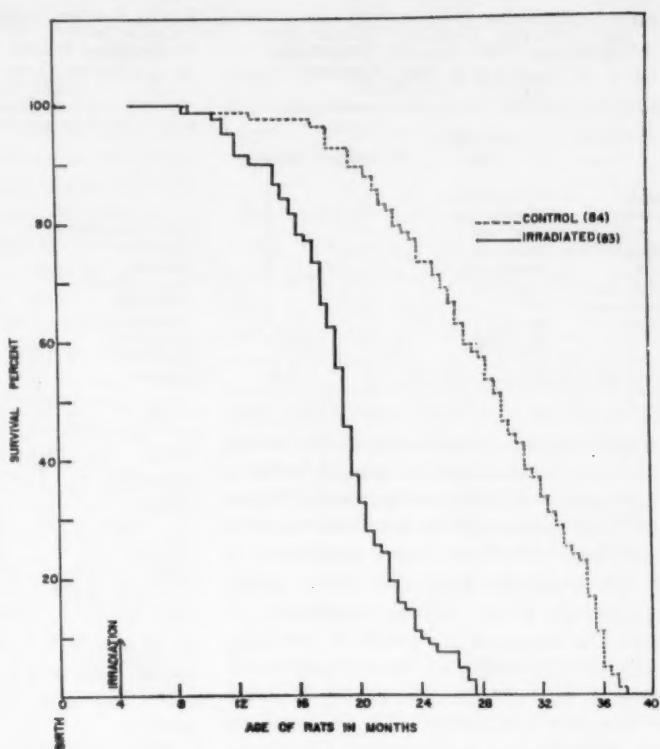
First, data from 167 survivors of a very large dose of *total-body* irradiation, 1,000 r, are presented in order to observe a maximal effect. In these initial studies the irradiation was administered while the female Wistar rats were exposed to a 5% O<sub>2</sub>, 95% N<sub>2</sub> mixture. This is a protective device that, when introduced prior and during irradiation, ameliorates the acute effects of the radiation exposure and permits survival for observation of late radiation changes.<sup>2</sup> Without such a protective hypoxic state ordinarily no rats would survive the 1,000 r *total-body* exposure.

**Shortened Life Span.**—The irradiated rats have a shorter life span, as seen in Chart 1. At 24 months post irradiation all are dead. Their mean survival is 15.0 months following the irradiation, contrasted with the mean survival of the nonirradiated controls of 24.5 months. This represents a 39% shortening of the life span normally expected after the time of irradiation.

**Growth Retardation.**—In Chart 2, the mean body weights of the survivors among the same group of rats are shown. This species normally continues to increase in weight throughout the greater portion of

DISEASES FOLLOWING X-IRRADIATION

Chart 1.—Survival of 84 control and 83 irradiated Wistar rats following 1,000 r hypoxic total-body irradiation in a single exposure at age 4 months.



its life span. This capacity to grow has also been reduced by the radiation exposure.

*Cause of Death.*—Autopsy studies and clinical observations in 125 of these same

rats have demonstrated the cause of death in most cases as well as the incidence of tumors and other disease. With the notable exceptions of hypertension, nephrosclerosis, and certain tumors to be mentioned below, irradiated animals and nonirradiated controls die with the same diseases, present in similar frequencies in both groups. There are no specific tissue changes that can be characterized as unique delayed irradiation effects.

*Incidence of Neoplasms.*—The neoplasms of the irradiated animals also are similar in type and distribution to those found in nonirradiated aged controls (Table 1). The low incidence of pituitary adenomas in the irradiated animals was an unexpected finding that has not been repeated in more recent work.

The increase in ovarian tumors in the irradiated rats is not statistically significant in this group of animals. However, when this ovarian tumor incidence is pooled with

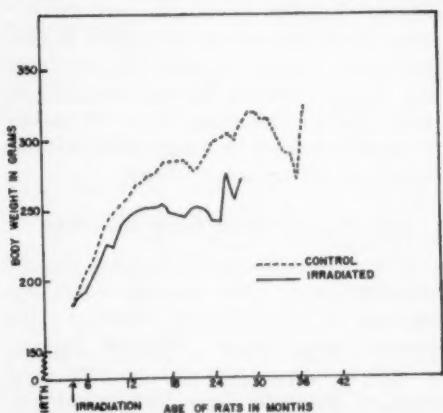


Chart 2.—Mean total body weight of surviving control and irradiated Wistar rats at monthly intervals following a single exposure to 1,000 r hypoxic total-body irradiation at age 4 months.

Lamson et al.

TABLE 1.—*Tumors at Death in Sixty-Four Control and Sixty-One Irradiated Wistar Rats Following 1,000 r Hypoxic Total-Body Irradiation in a Single Exposure at Age Four Months*

Tissue of Origin	Histologic Type	Irradiated	Control
Breast	Fibroadenoma	14	34
Ovary	Benign	8	3
Uterus	Malignant	0	3
Pituitary	Adenoma	1	21
Lung	Malignant	5	6
Disseminated	Malignant	4	1
Lymphoma		0	4
Others	{ Benign	8	5
	{ Malignant	3	4
Total Number of Malignant Tumors		12	18

similar data from other experiments an increase of ovarian tumors appears to be a specific delayed radiation effect in the Wistar rat. This radiation effect in rodents was first recorded by Furth in studies with mice.

It will also be observed in Table 1 that lymphomas which include leukemias are absent in this particular group of irradiated animals. Our strain of Wistar rats is not afflicted with a high spontaneous incidence of leukemia, and radiation has not increased the frequency of the disease. This is in contrast to studies in mice which indicate that radiation increases the incidence of leukemia even in those inbred strains with a low natural incidence of the disease.<sup>8</sup>

*Time of Appearance of Neoplasms.*—By the 24th month post irradiation, at which time all the irradiated rats are dead, more neoplasms have been found at autopsy in the irradiated rats than have been found in the smaller number of control rats that have also died. However, by the 34th month of the study, when all the controls have died and a final comparison of tumor incidence is possible, neoplasms found in the controls exceed those found in the irradiated group. In other words, at this dose level of 1,000 r with hypoxia, which is roughly comparable to 500 r without hypoxia, radiation accelerates the time of onset but does not increase the final incidence of malignancy—at least as observed in this strain of rat under the conditions of our laboratory.

TABLE 2.—*Age-Specific Incidence of Common Tumors in Sixty-Three Control and Fifty-Nine Irradiated Wistar Rats Following 1,000 r Hypoxic Total-Body Irradiation in Single Exposure at Age Four Months*

	Age at Death, Mo.			
	16-22	23-28	29-34	35-37½
Breast, fibroadenoma				
Irradiated	12	2		
Control		3	15	16
Lung, malignant				
Irradiated	2	3		
Control	2	1	2	1
Ovary, benign				
Irradiated	6	2		
Control		1		2

This acceleration of time of onset of benign and malignant neoplasms is demonstrated in Table 2. Through age 28 months, two years after the date of irradiation, the two benign and one malignant tumor types chosen for comparison are observed in greater numbers in the irradiated rats. When the tumors arising at later dates, up to age 37.5 months in the control rats, are included, only the benign ovarian tumors of the irradiated rats appear in greater final numbers.

The only other diseases observed with increased frequency in our Wistar 1,000 r hypoxic totally irradiated rats is a high incidence of hypertension and related nephrosclerosis. Autopsy data to date have revealed no lesion in the irradiated rats not observed at least on some occasion in non-irradiated controls of the same rat strain. The severe atrophy, fibrosis, bizarre epithelial changes, and telangiectases of vessels, so characteristic of local high-dose radiation injury, are conspicuously absent.

#### Effects of Partial Body Shielding

It is important to know what delayed effects will result from exposure of less than the total body to ionizing radiation. The delayed local changes following high-intensity therapeutic exposure of very small areas of the body have been mentioned. Patients receiving such doses unfortunately usually suffer from malignant disease. The high mortality usually associated with malig-

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nancy has provided little opportunity to study delayed radiation effects other than local tissue changes following this type of radiation exposure. Published data following experimental partial-body radiation exposures with doses comparable to those that can be withstood even when delivered to the entire body are very meager. Knowledge of the delayed effects following such limited exposures is important, for human radiation hazard is not always directed to the total body. Partial-body shielding has substantially reduced acute x-ray mortality in the lethal-dose range, particularly if a portion of the bone marrow has been shielded. Does such shielding also comparably ameliorate the delayed radiation-effects syndrome?

A recent publication<sup>4</sup> indicates that radiation of the abdomen alone, with the rest of the body shielded, results in substantial decrease of longevity. Our own data are limited to the 179 rats, shown in Table 3.

TABLE 3.—*Survival of Control and Irradiated Wistar Rats Following a Single Exposure of 1,000 r Partial- or Total-Body Hypoxic Irradiation at Age Three to Four Months*

Region of Body Exposed	Irradiation			
	Body Exposed, %	Dose, Gm.-r $\times 10^4$	Rats, No.	50% Survival, Mo.
Total body	100	1.83	26	12.0
Entire body, except head	88.2	1.61	32	13.5
Entire body except upper abdomen	81.9	1.50	38	14.5
Upper abdomen only	18.1	0.33	36	17.5
None, controls	0	0.00	47	22.0

Life span is similarly shortened following irradiation of the upper abdomen with 1,000 hypoxic r, although not significantly so, tending to confirm the data of the Belgian workers.<sup>4</sup> Also, it appears possible that there may be a gram-roentgen relationship present. Life-span shortening may be related in some fashion to the quantity of tissue radiated. Most probably, selected regions of the body will also be found that are sensitive to delayed radiation injury (as measured by shortened longevity) to a de-

gree out of proportion to the weight of the tissue involved. At any rate, it is not necessary to irradiate the entire body in order to produce a shortening of life span, and the upper abdomen is possibly a particularly sensitive region of the body.

#### Threshold for Delayed Somatic Injury

Our animal data so far reviewed have dealt with survivors of truly large total-body or partial-body radiation doses far above any expected exposure of our population not associated with mishap or nuclear war. Enumeration of the delayed effects following such large doses may indicate what smaller effects to search for following much smaller exposures. Herein lies one of the largest controversial problems facing students of radiobiology. *Do smaller radiation doses likewise exert a comparable delayed somatic effect, reduced in magnitude only in proportion to the decrease in size of the radiation exposure?* Or is there some threshold below which radiation exposure leaves no residual injury manifest at some later date, as, for example, a shortened life span? The matter is of considerable importance; for if no threshold exists, then all irradiation should be considered a life span-shortening agent. This shortening would be related in some fashion to the magnitude of the dose and after very small doses of several r, for example, could be very small indeed. Even our present so-called "permissible" radiation exposure and naturally occurring radiation would be expected to shorten life span by a small but definite amount.

At the present time there are convincing published data demonstrating life shortening in the rat following 150 r in a single exposure.<sup>5</sup> With exposure levels below 150 r, there is little available information, the existing scanty data largely derived from the mouse. A recent paper by Storer<sup>6</sup> indicates a straight-line radiation-dose life-shortening relationship that holds for x-ray doses between 0 and 100 rad, without apparent threshold. This paper provides a

useful reference list of publications bearing on radiation and longevity.

Most of the additional data on the effects of low levels of radiation upon longevity relate to chronic fractionated radiation exposures continued for the life of the animal. Results from experiments of this type have been contradictory. Some such studies have not included contemporary controls. In some others, controls have failed to live a normal life span. Furthermore, in all studies of this type much radiation is "wasted," in that many animals continue to receive radiation after they have already accumulated a life-shortening dose.

Many extraneous factors in the environment may possibly influence longevity. We are reluctant to rely upon longevity data unless the control group has attained a reasonable old age for the species concerned. Controls and irradiated animals must also share the identical environment and be observed concurrently. Longevity

comparisons in man which fulfill these requirements are obviously difficult to obtain.

Unfortunately, animal data sufficient to determine conclusively the effects of 100 r or less upon longevity are not available. Our own data on this problem in the lower-dose range (Chart 3) are still incomplete. We have observed a colony of 451 rats, including appropriate controls, for a period of 20 months after total-body exposures of 480, 240, and 120 r. Life shortening is sufficiently evident following 120 r so that one would predict with a fair degree of assurance that the shortening effect should be observable at substantially lower levels. If a threshold does exist, below which radiation has no effect upon longevity, one would hardly expect to observe a large effect at 120 r and no effect at all at, for example, 80 r.

The conditions of delivery of the radiation in these experiments at the 480, 240, and 120 r dose levels are pertinent. At each one of the three dose levels one group of rats has received the entire dose in a single exposure. Other groups have been exposed to the total dose divided in three or six equal fractions at either 3.5-day, 7-day, or 14-day intervals. Table 4 illustrates this fractionation at the 120 r level. As predicted by Blair,<sup>7</sup> fractionation of a 120 r dose results in life shortening comparable to that observed following a single 120 r

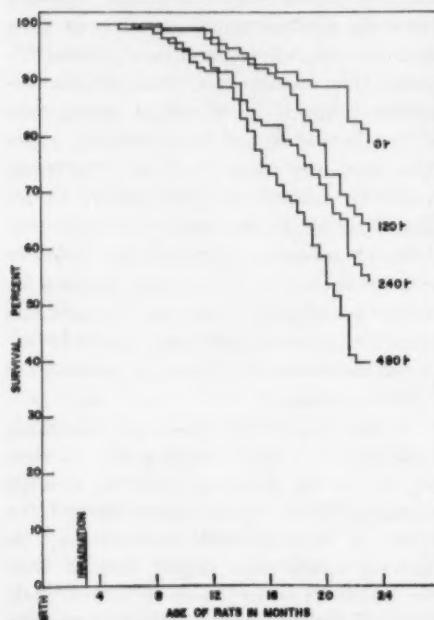


Chart 3.—Survival of 80 control and 371 irradiated Long Evans-Wistar hybrid rats 20 months after receiving 120, 240, or 480 r total-body irradiation in a single exposure or in divided exposures within a 10-week period.

TABLE 4.—Mortality of Eighty Control and One Hundred Twelve Irradiated Long Evans-Wistar Hybrid Rats Twenty Months After One Hundred Twenty r Total-Body Irradiation in a Single Exposure or in Divided Exposures Within a Ten-Week Period

Total Dose, r	Fractionation	Time Interval, Days	Initial No.	Conditions of Irradiation		Mortality—20 Mo. Postirradiation
				No.	%	
120	40 r×3	3.5	16	7	44	
120	20 r×6	3.5	16	6	38	
120	40 r×3	7.0	16	6	38	
120	20 r×6	7.0	16	3	19	
120	40 r×3	14.0	16	7	44	
120	20 r×6	14.0	16	7	44	
120	None	—	16	5	31	
0	—	—	80	17	21	

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exposure. Even six separate exposures of 20 r each at two-week intervals has hastened mortality to a degree comparable to the effect observed following the single exposure of 120 r. Possibly the time interval of two weeks between 20 r exposures has some significance.

Residual radiation damage following total-body exposure has been measured by testing the sensitivity of surviving animals to the acute effects of a second similar radiation exposure. When this is done it is usually found that survivors of one acute radiation exposure are less resistant to the second exposure. However, their resistance is partially and progressively restored as a greater period of time elapses following the initial radiation. By the use of this second-exposure device a residual subclinical injury can be demonstrated that apparently does not repair even after a long period.

In the rat the time for recovery of 50% of this reduced resistance is approximately five days. In two weeks approximately 87.5% of the reduced resistance has been regained. The irreparable damage that is measured by this residual 12.5% reduced resistance to acute radiation may or may not be related to shortening of life span. At any rate, it is of interest that six 20 r units of radiation, spaced close to this three half-life time interval of 15 days each, apparently adds an increment of irreparable damage. This damage is cumulative and is eventually measured as shortened life span, comparable in magnitude to that observed after 120 r in a single exposure. It appears reasonable to wonder if a *total* dose of 20 r in a single or divided exposure might not also leave a proportional residual stigma detectable in later life as shortening of life span. We believe these fractionation data, while by no means conclusive, strongly suggest that a longevity threshold, if such threshold exists at all, is probably below the 20 r level. In view of the possibility of reduced longevity following such small single doses as 20 r and in the absence of any really substantial data in favor of the existence of a threshold dose below which no

residual life shortening damage occurs, it appears prudent in our preventive medicine thinking to assume that no longevity threshold exists, until someone can conclusively show that it does exist.

*Threshold for Induction of Neoplasms.*—It has been mentioned that, in general, neoplasms appear sooner in irradiated rats, possibly as a part of an accelerated aging process. If there is no threshold for radiation effects upon longevity, then there may also be no threshold for this general acceleration of neoplasia.

The problem of radiation induction of neoplasms is, however, obviously complicated by additional considerations than accelerated aging alone. For example, several tumor types of endocrine origin, including the ovarian tumors already mentioned, have been observed in irradiated animals. It has been shown that some of these neoplasms are the result of radiation-induced endocrine imbalance acting upon an irradiated organ rather than the sole product of direct local effects of radiation upon the organ of tumor genesis. It is entirely plausible, from the work of Furth and others, that a threshold degree of hormone imbalance may be required in some cases to initiate the neoplastic process associated with radiation injury. Leukemia may be an additional neoplasm influenced by these endocrine considerations.

It should be apparent that immediate direct effects upon tissues exposed to radiation ionization could be proportional to the size of the radiation dose, no matter how small, as favored by most geneticists, without excluding the possibility of the existence of threshold states for various secondary manifestations which are dependent in addition upon the total body economy. We have many examples of such secondary threshold states in medicine. For example, a progressive lack of insulin may result in a proportional rise in blood sugar levels, and yet glucose does not appear in the urine in appreciable quantities until the renal threshold is exceeded.

### **Relationship of Acute Radiation Illness to Delayed Somatic Effects**

In spite of considerable biochemical and physiological data related to the effects of ionizing radiation on living tissue, the exact mechanism of acute radiation injury at the cellular level is still not known. The manner in which residual radiation-induced injury becomes manifest in later life as delayed somatic radiation effects is almost a complete enigma. The meager data available do indicate, however, that in a given species under certain conditions there is some relationship between the magnitude of acute and delayed effects. An agent, such as hypoxia, which reduces the severity of acute bone marrow destruction, ameliorates the immediate acute radiation illness generally, and reduces mortality also proportionately lessens the delayed radiation effects, as measured by life shortening. This observation does not necessarily imply that the delayed radiation effects are a direct consequence of the demonstrable tissue injury of the bone marrow and lymphatic tissue, for example, observed during acute radiation illness. It suggests rather that the sensitivity of the individual to acute radiation illness after large doses is also a measure of a related or parallel poorly understood mechanism of injury that later becomes manifest as shortened longevity.

Specific data on longevity of radiated animals assisted by other protective devices either before or after radiation exposure are not yet adequate. It might be expected, however, that procedures which are reparative rather than dose-modifying devices will not lessen the eventual development of delayed somatic radiation effects. The tumor data of Brecher<sup>8</sup> and Binhammer<sup>9</sup> are consistent with this belief.

### **Age Dependence of Radiation Effects**

It has been mentioned earlier in this discussion that the response to acute radiation exposure in the sublethal range is very similar in all mammalian species studied, including man. This fact becomes very

significant when radiation damage in laboratory animals is evaluated in terms of age at the time of irradiation. It has been shown that the acute LD<sub>50</sub> dose for a given animal strain is dependent upon the degree of maturity or age of the colony.<sup>7</sup> Young adult rats are maximally resistant to acute radiation injury. Immature developing animals are much more sensitive. The LD<sub>50</sub> dose for the rat at age 3 to 4 weeks is, for example, roughly half that observed at 4 months. Just how sensitive newborn rats may be at all stages of postpartum development is not yet clearly known. It should be emphasized that practically all the experimental data pertaining to somatic late effects have been accumulated after the irradiation of young adult laboratory animals. Assuming that the relationship between the magnitude of acute and delayed effects, discussed in the preceding section, is sustained by further experience, then we would expect that immature developing patients will also be much more sensitive to delayed somatic damage than is presently predicted from existing data. Direct experimental evidence relating age at exposure and severity of delayed somatic effects is now being accumulated in this and, no doubt, in other laboratories. Until experimental data indicate otherwise, it appears wisest to assume that young developing animals will be more sensitive to delayed radiation changes, such as shortened longevity, than fully mature animals. The possible implication of this reasoning upon the evaluation of hazards in pediatrics exposures will be mentioned below.

### **Evidence of Late Radiation Effects in Man**

The great bulk of the vast literature on radiation effects on man relates to the local changes following high-intensity limited-body exposure. No effort will be made to review this literature. Only a few pertinent papers, more directly related to the possible delayed somatic effects of total- or partial-body exposures of the magnitude under

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consideration in the experimental work of this report, will be considered here.

*Reduced Longevity.*—As already mentioned, the sensitivity of man to acute radiation hazard appears comparable to other mammalian species. The principal delayed general somatic effect of radiation exposure appears from animal data to be a decrease in longevity. If these animal studies may be extrapolated to man, on the strength of this similarity in acute response, shortened longevity in man should be a most plausible possible effect. Obviously, length-of-life differences must be large in order to draw valid conclusions from relatively small groups of humans with varying age distributions living in uncontrolled and heterogeneous environments. The shortening of life span of radiologists, as compared to nonradiologists among the medical profession, for example,<sup>10</sup> has not been considered entirely satisfactory evidence because of the possibility that other variables in the environment may also have influenced longevity.<sup>11,12</sup> Valid comparisons of longevity between two otherwise similar groups of humans, each with known levels of radiation exposure, are difficult to obtain except under very unusual conditions. Longevity statistics from atomic bomb survivors in Japan may eventually provide such information.

A very slight retardation of growth has already been observed among children exposed to radiation in the Rongelap Marshall Islands as a result of the 175 r total-body exposure from radioactive fallout.<sup>13</sup> From our rat data we would expect this small decline in growth vigor to be associated with a shortened life span as well. Many years must yet pass before the necessary data from this area are available. The small numbers of exposed persons involved in this Marshall Island mishap will possibly prevent statistically valid conclusions.

*Incidence of Malignancy.*—A radiation-dose-related high incidence of leukemia has already been observed in Japanese survivors of the wartime bombings.<sup>11</sup> The incidence

of leukemia among United States radiologists has also been cited as evidence of delayed radiation damage.<sup>10,11</sup> However, only 17 deaths from leukemia were recorded in the latter group between 1938 and 1952.<sup>11</sup> Some have argued that this small number of leukemia cases in United States radiologists indicates a low level of late radiation effects in this group of medical specialists. This incidence of leukemia among United States radiologists may well indicate a much lower level of irradiation exposure than in many of the Japanese bomb survivors. It does appear certain, however, that leukemia can be increased in frequency by radiation in man.

If we can extrapolate from data derived from rats, we might also profitably search for evidence of a larger age-specific incidence of other common human malignancies and other age-related diseases in populations suspected of experiencing a significant radiation exposure. If one were to compare only the absolute prevalence of neoplasms in general, without regard to their time of occurrence, one might conceivably overlook significant postradiation effects. The prevalence of cancer on the whole among our irradiated rats is not greater than in the controls. The cancers have in general, however, appeared at an earlier age. Warren has studied the age at death of radiologists and other physicians afflicted with various malignancies, including leukemia, and other diseases. All causes of death appeared sooner among the radiology group.<sup>10</sup> This type of comparison is less sensitive to distortion by variation in age distribution between the two groups than a simple comparison of mean age at death.

*Radiation and Malignant Disease in Children.*—Recently an increased incidence of cancer of several types, including leukemia and thyroid cancer in children having received x-irradiation to the thorax for enlarged thymus, has been recorded.<sup>14</sup> One cannot be sure that the malignant disease was produced by the irradiation in every case, but it is suggestive that some of these children with tumors had received only

200 r. The publication by Stewart,<sup>15</sup> describing an increase of leukemia and other tumors in children, who received very small doses of total-body irradiation while in utero during maternal pelvimetry, also awaits confirmation. This small bit of evidence relating to the consequence of irradiation in children gives some support to our thesis, developed above, that immature developing individuals may be more sensitive to somatic delayed radiation effects, as measured by longevity and time of appearance of neoplasms, than young adult individuals.

#### Possible Hazards from Diagnostic X-Ray Use

Hursh, in a published lecture, computes that the "permissible" dose of 0.3 r per week total-body exposure to a *young adult* for 20 years conceivably might reduce life span by 2 years out of 65.<sup>16</sup> This is a life shortening of approximately 2.3 days for each roentgen of total-body radiation. This calculation assumes that no threshold for reduced longevity exists and that animal data may be extrapolated to man, with adjustments for the difference in life span in the two species. It is an as yet unsupported calculation, deliberately representing the worst possible combination of assumptions based on animal data. More recently Failla<sup>17</sup> has made calculations dependent upon similar assumptions and has arrived at a slightly smaller life-shortening figure of approximately one day per roentgen.

An amount of 0.3 r per week is roughly 15.6 r per year. How does this compare with the magnitude of radiation in typical diagnostic procedures? The Radiological Health Branch of the United States Public Health Service lists the following average exposures: radiographic examination, 2.7 r; photofluorographs, 1.0 r, and fluoroscopic, 65.0 r. If one converts these skin doses to average depth dose by using a factor of 0.1, a single fluoroscopic examination should deliver approximately 6.5 r partial-body radiation. This 6.5 r represents total accumulated dose for the examination. In a properly performed fluoroscopic procedure

only a small area is exposed at one time. Many segments of the examined area, varying in size from  $5.0 \times 5.0$  cm. to  $10.0 \times 10.0$  cm., should possibly receive only 0.1 of this total dose, or 0.65 r. Recent technical improvements, such as faster films, higher voltages, more filter, and better coning, should also reduce this figure even lower.

It has occasionally been argued that since the majority of medical x-ray diagnostic exposures are such partial body exposures, they should not be compared with effects from total-body exposure in animals. Support for this concept is gained from the work of Kaplan<sup>18</sup> and Lorenz<sup>19</sup> in mice, demonstrating that radiation-induced leukemia can be inhibited by partial shielding of the body. Recent data, however, such as have been summarized here, indicate that effects on longevity, although proportionately reduced by partial shielding of the body, are not eliminated by this procedure. Partial-body radiation cannot be disregarded as a potential hazard on the grounds alone of failure to irradiate the entire body.

Our animal data also suggest, moreover, that irradiation of the upper abdomen alone may produce life shortening greater than the proportional volume of tissue involved. If we assume that irradiation to the upper abdomen is only one-third as hazardous as the same number of r delivered to the total body, then a gastrointestinal fluoroscopy might be equivalent to approximately 0.2 r of total-body irradiation, based upon the preceding calculations. Again, assuming no threshold for radiation effects upon longevity and that animal data can be safely extrapolated to man, on the basis of the calculations of Hursh, 0.2 r total-body irradiation might reduce our life span on the average by only a half day. When one compares the possible benefit to be gained by properly indicated and conducted medical examination of this type, a hazard of this small size from a gastrointestinal fluoroscopy becomes negligible.

On the other hand, there is possible danger in failing to recognize a very small risk or in deliberately saying that a very

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small risk is no risk at all. If x-ray at the diagnostic level is harmless, it can be used at will without restraint. Furthermore, this estimate of minute life shortening following a typical diagnostic fluoroscopic examination assumes proper technique in the performance of the procedure as well as the use of modern well-calibrated equipment. Outdated methods or careless practices could substantially increase the radiation exposure by many fold.

We believe that existing data are not adequate to know with certainty that very low x-ray doses are entirely harmless. We think there is sufficient evidence from animal data to suspect that all radiation, even in very small doses, may have some biological effect. The existing evidence suggests that children may be more sensitive to this effect than mature persons. We favor the continued careful use of properly indicated diagnostic medical x-ray procedures by competent personal, versed in monitoring procedures and using the most modern improved technique. Such persons are in the best position to define and compare the very small risk involved against the much greater probability of benefit to be gained.

Department of Pathology, School of Medicine, University of California Medical Center (24).

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# Experimental Myocardial Infarction in the Rat

NATHAN KAUFMAN, M.D.; T. L. GAVAN, M.D., and R. W. HILL, M.S., Cleveland

Sequential morphologic and histochemical changes induced in the rat myocardium after coronary artery ligation are considered in this report. This study forms the basis for a series of experiments to investigate factors producing and modifying histochemical changes in the myocardium associated with coronary artery ligation. Since large numbers of readily available animals of uniform stock are desirable in the design of these experiments, it is necessary to use an animal such as the rat for this purpose.

## Material and Methods

Adult male albino rats (250-300 gm.) of the Sprague-Dawley strain were used. The left coronary artery was tied according to the method of Johns and Olson.<sup>1</sup> Under light ether and sodium pentobarbital anesthesia (2.8 mg. per 100 gm. of body weight), a left thoracotomy was performed through the fourth or fifth intercostal space. The pericardium was exposed and incised. A small curved round needle carrying an artificial nonabsorbable surgical suture, 000000, (silk) was passed through the ventricular myocardium at the tip of the left auricular appendage to occlude the left coronary artery, which is embedded in the myocardium at this point. The thoracic wall was then closed with 00 chromic gut suture, and the skin approximated with linen suture. While the thoracic cavity was open, respiration was maintained with positive pressure 90% oxygen and 10% CO<sub>2</sub>, administered by means of a tight-fitting face mask. After surgery, the rats were placed in an atmosphere of 90% O<sub>2</sub> and 10% CO<sub>2</sub> until they had recovered sufficiently from an-

Submitted for publication Sept. 30, 1958.

This investigation was supported by a grant from the Cleveland Area Heart Society.

From the Department of Pathology Cleveland Metropolitan General Hospital (formerly Cleveland City Hospital) and Western Reserve University School of Medicine; Research Fellow, National Heart Institute, Public Health Service (HF-6624) (Dr. Gavan).

esthesia to move about freely (10-30 minutes). Animals with infarcts were autopsied after intervals of 2, 4, 6, 12, 18, 24, 36, 42, 48, 72, and 90 hours. There were at least four rats in each group after coronary artery ligation. Animals which underwent a similar surgical procedure, except that the ligature was placed adjacent to rather than about the coronary artery so as not to occlude the vessel, served as controls. In addition, the myocardium in those portions of the heart which were not involved in the infarct acted as an internal control in each animal. In many of the animals, electrocardiograms were done to determine whether or not infarction had occurred before gross morphological changes were expected.

Thin tissue slices of the heart from each animal, including left and right ventricular walls and interventricular septum, were placed in 10% neutral formalin, in cold acetone-alcohol (1:1) mixture for alkaline phosphatase, and in cold Bouin solutions for glycogen stains. Similar slices were kept fresh for frozen section. The histologic and histochemical procedures were hematoxylin and eosin stain, the reactions for succinic dehydrogenase,<sup>2</sup> cytochrome oxidase,<sup>3</sup> alkaline phosphatase,<sup>4</sup> the periodic acid-Schiff (PAS)-diastase method for glycogen, and oil red O and Sudan IV for fat.

The amount of succinic dehydrogenase and cytochrome oxidase activity present in the infarcted myocardium was estimated by comparison with the activity in a corresponding area in the operated control hearts.

## Results

No demonstrable change in succinic dehydrogenase activity was seen in two hours. By four hours, a change in the enzymatic activity was seen in one of four rats. The intense purple precipitate was partially replaced by blue-red granules. No decrease in the quantity of precipitate was observed. By six hours, the change in color was more pronounced in all of the infarcts. In 12 hours, there was a definite reduction in precipitated granules, which was more pronounced at 18 hours (Fig. 1). Only traces

## EXPERIMENTAL MYOCARDIAL INFARCTION

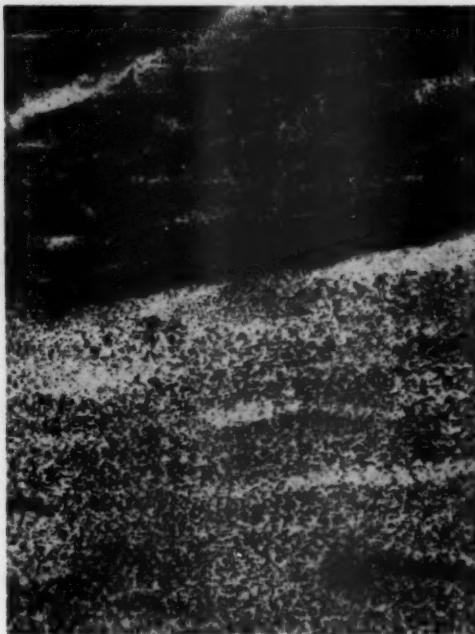


Fig. 1.—Succinic dehydrogenase activity in an 18-hour infarct. Note the decreased enzymatic activity in the lower infarcted portion, when compared with the non-infarcted myocardium above. Neotetrazolium method;  $\times 100$ .

of activity were seen in the infarcted areas at 24 hours. Areas of no enzymatic activity in the infarcts were seen in 48, 72, and 90

hours (Fig. 2). Cytochrome oxidase activity was initially decreased in infarcts in 12 hours, and by 72 hours no activity was

Fig. 2.—Succinic dehydrogenase activity in a 72-hour infarct. Normal enzymatic activity in the uninfarcted myocardium is seen in the upper right hand corner. Complete loss of activity is evident in the infarcted myocardium. The few dark-staining granules in the infarct represent fat in macrophages. Neotetrazolium method;  $\times 100$ .

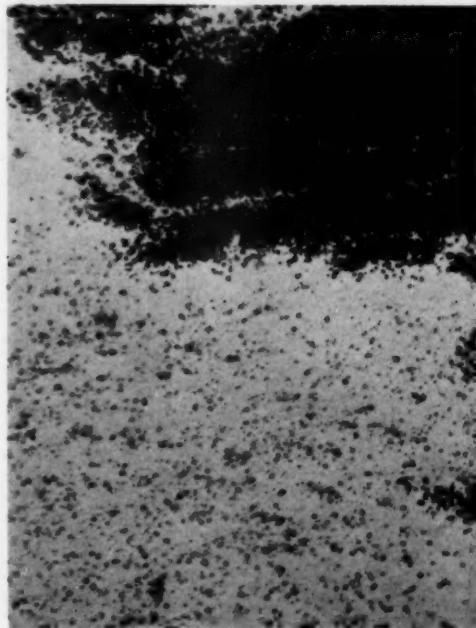
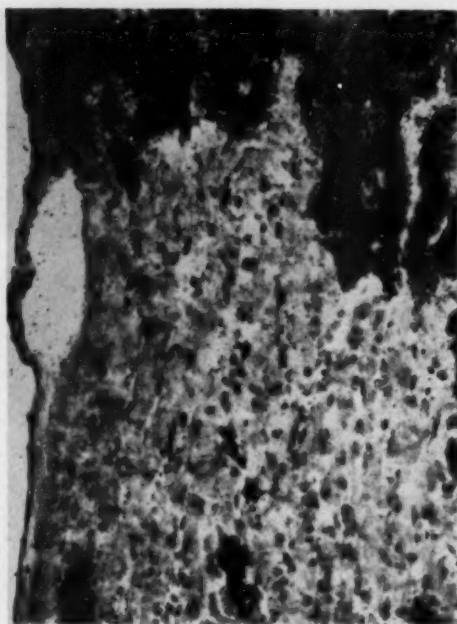


Fig. 3.—Fat in an 18-hour infarct. The central area of the infarct contains very little fat. Most of the fat is deposited at the periphery of the infarct. Oil red O method;  $\times 180$ .



seen in most of the infarcted area. There was no alteration of enzymatic activity in the controls.

Fatty degeneration of myocardial fibers was first observed four hours after coronary artery ligation. By 6 hours and sim-

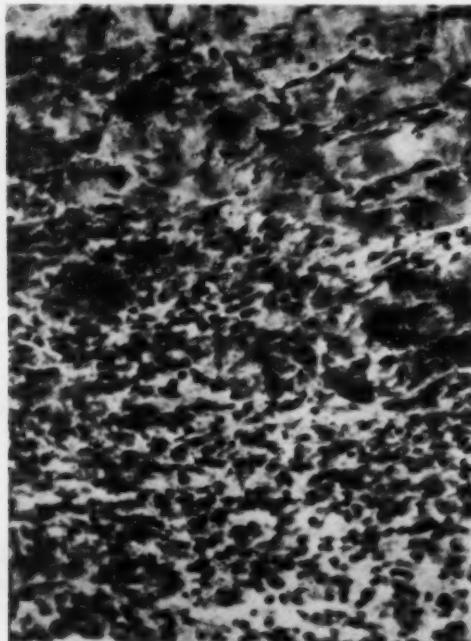


Fig. 4.—Fat stain in 90-hour infarct. There is a decrease in fat in the myocardial fibers at the periphery of the infarct in the upper portion. The fat seen in the infarct below is contained within macrophages of the infiltrate. Oil red O method;  $\times 220$ .

## EXPERIMENTAL MYOCARDIAL INFARCTION

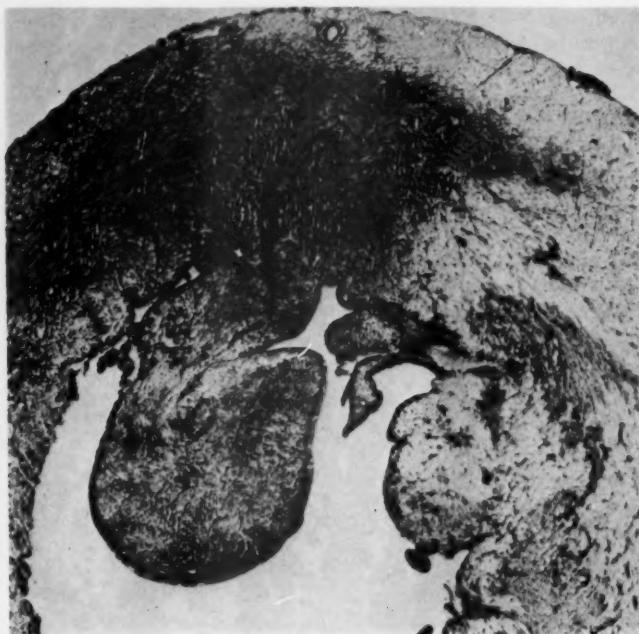


Fig. 5.—(PAS) periodic acid-Schiff-positive reaction in 18-hour infarct. Glycogen is seen in the noninfarcted myocardium in the upper portion of the illustration. The PAS-positive material in the adjacent infarcted myocardium, below and to the right, does not represent glycogen, since it is diastase resistant (Fig. 6). Periodic acid-Schiff;  $\times 17$ .

ilarly at 12 hours, fatty changes were very marked and definite, particularly in the peripheral portions of the infarct. As the

central area of the infarct became more necrotic fat droplets disappeared from the necrotic fibers between 18 and 24 hours,

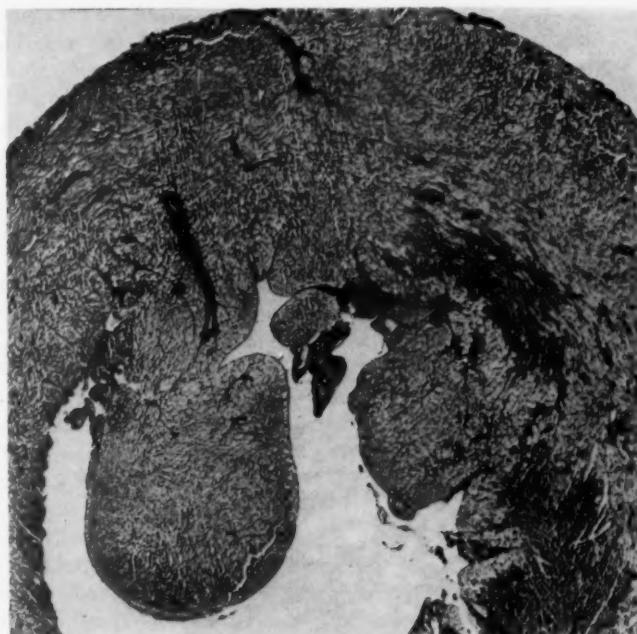


Fig. 6.—PAS-diastase reaction in 18-hour infarct. The glycogen seen in the noninfarcted portion of the myocardium shown in Figure 5 has been digested, leaving only the PAS-positive diastase-fast material in the infarct. Periodic acid-Schiff-diastase;  $\times 17$ .

Fig. 7.—Infarct, 24 hours after coronary artery ligation. The cellular infiltrate consists of polymorphonuclear leukocytes and mononuclear cells. Note the swelling and vacuolization of many myocardial fibers. Hematoxylin and eosin;  $\times 360$ .



leaving the fat in the periphery (Fig. 3). The number of fat droplets at the periphery increased to a maximum after 18-24 hours

of ischemia and then decreased. By 90 hours, decrease in fat at the periphery of the infarct became more marked (Fig. 4).



Fig. 8.—Infarct, 90 hours after coronary artery ligation. The cellular infiltrate in the infarct (in the lower portion) consists chiefly of mononuclear cells and fibroblasts. Hematoxylin and eosin;  $\times 390$ .

## EXPERIMENTAL MYOCARDIAL INFARCTION

The fat seen in the infarcted area was in macrophages, whereas that in the periphery was present in the myocardial fibers.

It was difficult to ascertain the decrease of glycogen in the infarcted areas with certainty because of the erratic distribution of the small amount of demonstrable glycogen in rat myocardium. Infarcted myocardial fibers became PAS positive as early as 6 hours after coronary ligation in four of five animals and by 12 hours in all of the animals. The intensity of PAS-positive-staining material increased with time. This PAS-positive substance resisted diastase digestion and was nonmetachromatic when stained with toluidine blue (Figs. 5 and 6).

At six hours, definite interstitial hemorrhage was noted. A cellular infiltrate, consisting chiefly of polymorphonuclear leukocytes, did not appear in the interstitial tissues until after 18 hours, although prior to this time an increasing number of polymorphonuclear leukocytes were seen in the congested vascular spaces. Within 24 hours, a moderate number of mononuclear cells began to appear (Fig. 7) and by 72 hours predominated the cellular infiltrate surrounding the necrotic myocardial fibers. This was more pronounced at 90 hours (Fig. 8). Fibroblasts made their appearance in the cellular infiltrate after 36 hours and then became increasingly more prominent.

The myocardial fibers showed no apparent alteration before six hours. At this time, there was a slight increase in the eosinophilia of some fibers, with no alterations in cross striations. The myocardial fibers then became more eosinophilic, and necrosis with typical nuclear changes and loss of cross striations became evident by 18 hours.

Since biochemical determinations by others have shown that the heart contains alkaline phosphatase activity, sections of the myocardium were studied with use of the calcium-cobalt method for this enzyme.<sup>3</sup> This study confirmed the observation that histochemically demonstrated alkaline phosphatase activity is confined to the endothe-

lium of the interstitial capillary network and adventitia of moderate-sized arteries and is not noted within the myocardial fibers.<sup>4</sup>

### Comment

No morphologic or histochemical alterations were observed in the rat myocardium during the first two hours after coronary artery ligation. The earliest changes noted were in the color of the formazan precipitate in the succinic dehydrogenase reaction and the fatty degeneration of myocardial fibers in the region of the infarct at four hours. Cytochrome oxidase activity remained normal. No morphologic evidence of infarction could be noted. A definite decrease in succinic dehydrogenase and cytochrome oxidase activity at 12 hours was seen in the infarct, with complete inactivation of these enzymes at 48 and 72 hours, respectively. These changes parallel the quantitative determinations of Jennings et al.,<sup>5</sup> who observed no loss of succinic dehydrogenase activity in the first 4 to 5 hours in the dog, but this was followed by a sharp decrease in activity which reached a 50% level in 12 to 15 hours.

Fatty degeneration was marked in myocardial fibers located at the periphery of the infarct. These fibers, although the site of fatty degeneration, gave a normal histochemical reaction for succinic dehydrogenase and cytochrome oxidase. In the central portion of well-established infarcts, where necrosis is definite and the changes are irreversible, very little fat or enzymatic activity was present. On the other hand, at the periphery both the fat and the enzymatic activity persisted for the duration of the experiment. This suggests that fatty degeneration in myocardial fibers is associated with reversible injury. Wartman et al.<sup>6</sup> arrived at the same conclusion on the basis of morphologic studies.

Morphologic changes, as observed in hematoxylin and eosin stained sections, were not apparent until after evidence of infarction had already been established with the enzyme reactions and fat stains. These

histologic alterations were similar to those described in the dog by Karsner et al.<sup>7</sup> and in the human by Mallory et al.,<sup>8</sup> the main difference being the rapidity with which these changes occurred. In the rat, the sequence of events was slightly more rapid than in the dog or the human. Mallory et al.<sup>8</sup> pointed out that the size of the heart may be the factor responsible for these species differences.

The evaluation of histochemically demonstrable glycogen in the infarcts is not as satisfactory as in the dog, since the distribution is random and the amount found in the rat myocardial fibers is less. This correlates with the evidence that the rat myocardium contains approximately one-third of the amount of glycogen found in the dog.<sup>9</sup> In spite of this irregular distribution of glycogen, none was seen in the areas of infarction (Figs. 5 and 6). Myocardial fibers in the infarcted area become PAS-positive after six hours of ischemia. In the dog, this change was first noticed at four hours by Kent and Diseker<sup>10</sup> and in the first few hours by Wartman et al.<sup>6</sup> The exact nature of this PAS-positive substance is now known. It is not glycogen, since it is diastase-fast, nor is it an acid mucopolysaccharide, since it gives a nonmetachromatic reaction when stained with toluidine blue.

### Summary

Myocardial infarcts were produced in rats, and histochemical and morphologic changes, observed at intervals up to 90 hours. An alteration in succinic dehydrogenase activity was first observed in 4 hours, with a definite decrease in 12 hours. By 48 hours, areas of no enzymatic activity were seen. Cytochrome oxidase initially decreased in 12 hours and disappeared

from the infarct in 72 hours. Fatty degeneration was first noted at four hours. Diastase-fast-PAS-positive material first appeared at six hours. The rat was found to be a satisfactory animal for the study of sequential histochemical and morphologic changes in the myocardium.

Department of Pathology, Cuyahoga County Hospital, 3395 Scranton Rd. (9).

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# Mechanism of Hematopoiesis

## *Hematopoietic Effects of Serum Albumin*

BERNHARD STEINBERG, M.D., Toledo, Ohio

### Introduction

It is almost an axiom that any function in the body does not take place in a void of haphazard series of acts and reactions but is subject to a rigid and disciplined control, which is interrelated to other functions. This concept applies equally well to hematopoiesis. Initiation of blood-cell production, selective maturation, delivery of specific numbers of cells into the blood stream, maintenance of cell ratios, cell aging, and time-spaced disintegration require regulation and coordination. The purpose of this and subsequent presentations as well as some previous ones<sup>1-4</sup> is to explore the nature of this regulatory mechanism of hematopoiesis.

In a previous article<sup>3</sup> investigations were described in which multiple intravenous injections of measured doses of either bovine or human serum into rabbits resulted in inhibition of maturation and production of cells in the bone marrow. Three possible explanations for these effects were considered. An antigen-antibody reaction in which the marrow was the shock organ was one. A second possibility was a "toxic effect" of proteins foreign to the rabbit with a specific action on the animal's marrow. The third explanation was the presence in the serum of regulatory factors with inhibitory functions. The demonstration of inhibitors instead of accelerators was con-

sidered to be due to the greater potency of the former.<sup>1</sup> On the basis of several observations, the last explanation was accepted, at least for the present, as the most plausible.

In a previous study,<sup>5</sup> it was pointed out that a single or even a few injections of a material with presumptive hematopoietic effects was insufficient in most instances to disturb the regulators in a normal animal. When attempts were made to give rabbits repeated injections of heterologous serum many of the animals died from vascular collapse after two to four injections. Further investigation succeeded in eliminating the "toxic factor" by dialysis of the serum.<sup>6</sup> Other observations indicated that the optimum time for injections was at 48-hour intervals and the most accurate timing for evaluating the cellular picture of the peripheral blood was just prior to the intravenous administration.<sup>5</sup> Several nonspecific and marrow expulsion activities interfere in the first 48 hours with interpretation of the changes in the peripheral blood.<sup>2</sup>

### Experimental Procedures

Since multiple intravenous injections of the whole normal human serum into rabbits resulted in inhibition of cell maturation and occasionally of cell production of the marrow, the next logical step was to fractionate the serum proteins to determine if this effect was due to the total protein or to individual components. Previously,<sup>4</sup> albumin was injected but no hematopoietic changes were noted. However, the albumin used at that time was prepared by the "cold alcohol" method, and in the light of the experiments presented now, the method of preparation is significant.

As the first step in this study, the serum protein of normal human serum was fractionated into albumin and globulins by continuous-flow electrophoresis. The Spinco model was used. The

Submitted for publication Oct. 20, 1958.

From The Toledo Hospital Institute of Medical Research.

This work was sponsored by a grant from the John A. Hartford Foundation, Inc.

Acknowledgment is made to Leon Libenson, Ph.D., for his aid in the continuous flow electrophoresis.

undiluted serum was fed at the rate of 1.5 ml. per hour at the top of the filter-paper curtain through which an electrical field is established.

The entire procedure was carried out in a refrigerated room at 3 C. The temperature within the electrophoretic cell was 8 to 9 C. The component fractions of the serum were separated and collected in tubes. The total volume of serum which was processed was 30 to 35 ml. per 24 hours. The fractions were dialyzed against distilled water at 6 C three times at 24-hour intervals. Paper electrophoresis was run on each component to establish the degree of separation, and the fractions were lyophilized. The fraction labelled CFE-4 contained the major part of the albumin. Fraction CFE-3 was composed of  $\alpha$ -globulin and albumin. Fractions CFE-2 and 1 were made up of  $\beta$ - and  $\gamma$ -globulins, respectively.

Twenty-five rabbits of the New Zealand White breed of either sex, between the ages of 2 and 4

months, were used. Intravenous injections were given once every 48 hours into the marginal ear vein. The dose consisted of 4.4 ml. in terms of original serum per pound body weight. Prior to the injection, blood was drawn from the ear vein for the total leukocyte, erythrocyte, thrombocyte, and reticulocyte counts. Slide preparations were made and stained with Wright's stain for differential study. Thrombocytes were counted by the Brecher method with the use of a counting chamber. Hemoglobin determinations were done with the photoelectric colorimeter and the hematocrit by the micromethod.

Each of the 4 fractions obtained by continuous-flow electrophoresis of normal human serum was injected into 5 rabbits, making a total of 20 animals. From 8 to 12 injections were administered. Five additional rabbits were given injections 8 to 16 times under similar conditions with normal serum albumin processed by E. R.

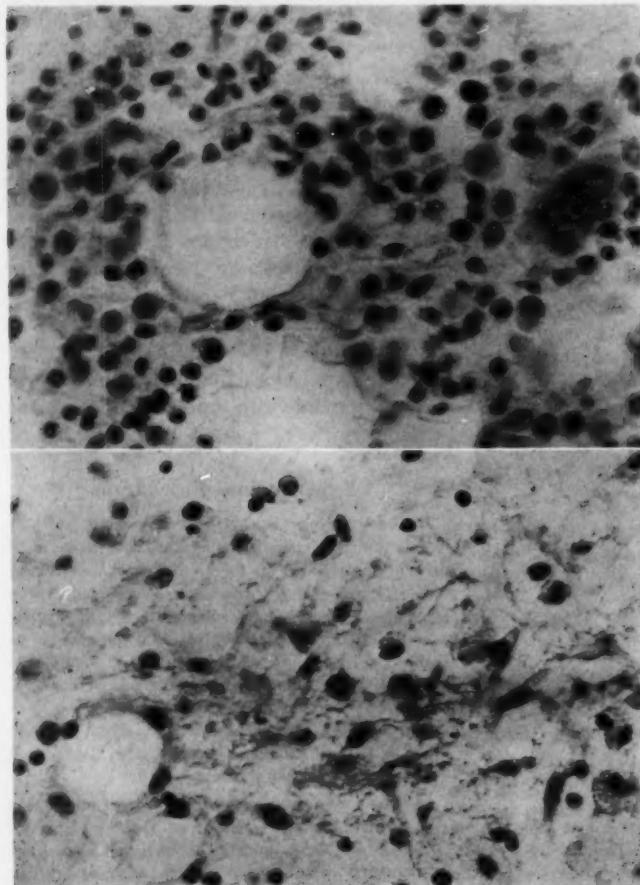


Fig. 1.—Bone marrow of a rabbit given eight injections of albumin obtained by continuous-flow electrophoresis from normal human serum. There is inhibition of cell production and maturation.

## HEMATOPOIESIS

TABLE 1.—Normal Human Serum Fractionated by Continuous-Flow Electrophoresis into Four Fractions and Injected Intravenously at Forty-Eight Hour Intervals into Twenty Rabbits Eight to Twelve Times—Blood and Tissue Studies\*

Fraction No.	Electro-photoretic Composition	Changes in Peripheral Blood	Changes in Marrow & Other Organs
1	$\gamma$ -Globulin	No changes except for leukocytosis of 16,000	Granulocytic hyperplasia of marrow
2	$\beta$ -Globulin	Hgb. from 11.6-8.3 gm.; leukocytes to 19,300	Granulocytic hyperplasia of marrow & hypoplasia of lymphoid tissue
3	$\alpha$ -Globulin	No changes except for lymphocytosis of 14,361	Hyperplasia of marrow granulocytes & of lymphoid tissue
4	Albumin	Leukopenia, granulocytopenia, thrombocytopenia, & anemia (see Table 2)	Marrow shows immaturity of granulocytes & erythroid cells & patchy areas of fibrosis; lymphoid tissue is immature

\* Interpretation: The albumin component of serum protein of normal human blood acts on the marrow of rabbits to produce inhibition of maturation of the marrow cells. This inhibition in the marrow is reflected in the peripheral blood.

Squibb & Sons from the blood collected and pooled by the American Red Cross and supplied by the Toledo chapter of the American Red Cross and its Director, Hoyt Meader, M.D. The albumin processed by Squibb was prepared by the cold alcohol method and sterilized by exposure to a temperature of 60°C for 10 hours.

TABLE 2.—Peripheral Blood Changes in Rabbit Following Injections of Serum Albumin of Normal Human Blood Fractionated by Continuous Electrophoresis.—Blood Studies Performed Forty-Eight Hours After Injections\*

Injections, No.	Hgb., Gm.	Erythrocytes Cu. Mm.	Hematocrit, %	Leukocytes Cu. Mm.	Differential Leukocyte Count			Thrombocytes Cu. Mm.	Reticulocytes, %
					Lymphocytes	Granulocytes			
Prior to injection	11.9	6.19	40	5,550	3,850	1,700	400,000	2.8	
1	11.3	5.72	38	11,450	3,192	8,258	380,000		
2	11.	5.89	38	10,500	7,035	3,465	315,000		
3	11.3	5.74	38	7,250	4,464	2,786	370,000		
4	10.4	5.54	35	10,650	6,572	4,078	340,000	6.9	
5	8.9	4.66	31	9,550	5,820	4,230	350,000		
6	8.	4.59	32	7,750	4,004	3,746	200,000		
7	8.	4.20	27	7,050	4,200	2,850	150,000		
8	5.7	2.70	17	3,450	2,958	492	140,000	1.1	
9	4.3	2.23	17	1,500	1,470	30	100,000	1.1	

\* Interpretation: Injection of serum albumin obtained by continuous-flow electrophoresis produces in rabbits considerable decrease of hemoglobin, hematocrit, and all the blood-cell components of the peripheral blood. This change is associated with inhibition of cell maturation and proliferation in the marrow.

## Results

The rabbits which were given injections with the albumin component of normal human serum obtained by continuous-flow electrophoresis showed inhibition of cell maturation and proliferation of the marrow (Fig. 1). The peripheral blood revealed a substantial decrease of all the cell components and especially granulocytes, with a reduction of hemoglobin content and hematocrit volume percentage (Table 2). The various globulin fractions, when injected into the rabbits, showed a persistent granulocytic hyperplasia of the marrow and a slight leukocytosis of the peripheral blood (Table 1). The leukocytosis was contributed for the most part equally by granulocytes and lymphocytes;  $\beta$ -globulin fraction produced a slight decrease of hemoglobin. Hyperplasia or hypoplasia of the lymphoid tissue obtained with the various globulin components was not sufficient to be significant. The other changes produced by the globulin fractions require further investigation and evaluation.

The animals which were given injections of the Squibb normal human serum albumin did not show any changes of the marrow, even after 16 injections (Fig. 2). The peripheral blood of the rabbits given injections of this albumin did not reveal

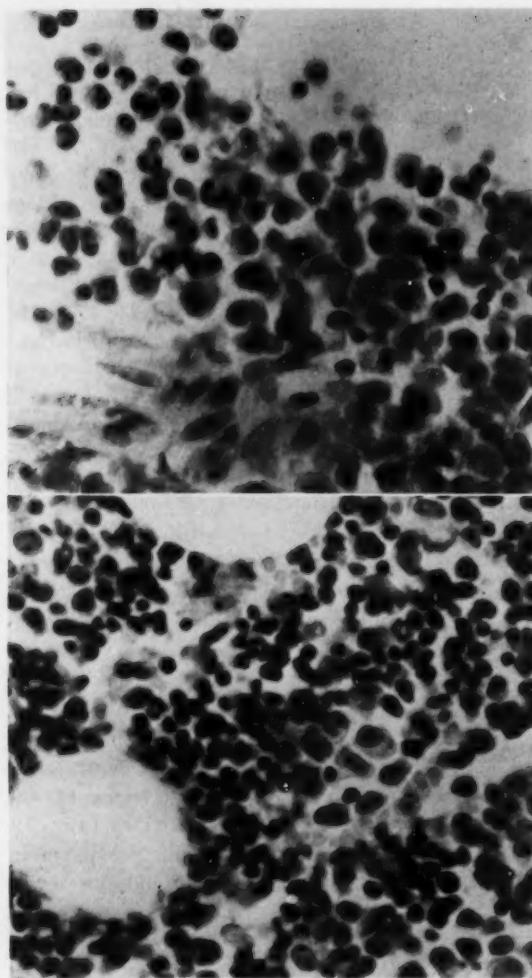


Fig. 2.—Bone marrow of a rabbit given 16 injections of albumin obtained from E. R. Squibb & Sons. The albumin was heated by the processor at 60 C for 10 hours and prepared by the "cold-alcohol" process. The marrow does not show significant changes from normal.

changes of the blood cells, hematocrit, or hemoglobin.

Paper-electrophoretic patterns were obtained on the blood of all the animals before, at intervals during the injection of the various fractions, and at the termination of each experiment on the rabbits. The patterns of blood of rabbits given injections of albumin obtained by continuous-flow electrophoresis showed a consistent decrease in the percentage of albumin, even before the appearance of hematological changes in the peripheral blood;  $\beta$ - and  $\gamma$ -globulins were elevated (Fig. 3). The total proteins were

increased by 1.5 to 2 gm. per 100 ml. of blood. On the other hand, the patterns of blood of rabbits given injections of albumin processed by Squibb did not reveal any changes (Fig. 3). However, the total proteins were increased by 1.5 to 2 gm. Those rabbits given injections of  $\gamma$ -,  $\beta$ -, and  $\alpha$ -globulin fractions of normal human serum obtained by continuous-flow electrophoresis showed a decrease in albumin and an increase in  $\gamma$ -globulins.

After the albumin was allowed to age for one year in a lyophilized state and in vacuo, the leukopenia factor was present, the an-

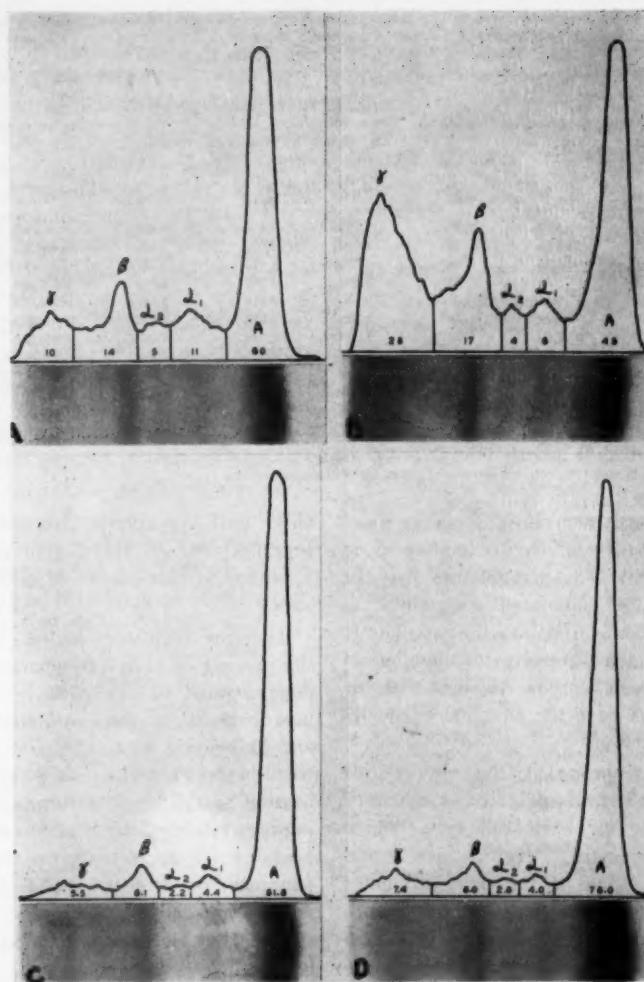


Fig. 3.—Paper-electrophoretic patterns of blood serum of rabbits given injections of albumin obtained from normal human serum by continuous-flow electrophoresis and patterns obtained after injection of albumin prepared by E. R. Squibb & Sons by the "cold-alcohol" method, followed by heating at 60°C for 10 hours. *A*, paper-electrophoretic patterns of a rabbit's blood serum before and *B* after eight injections of normal human serum albumin obtained by continuous-flow electrophoresis. The albumin was decreased by 15%;  $\beta$ - and  $\gamma$ -globulins were increased by 3% and 18%, respectively. *C*, paper-electrophoretic patterns of a rabbit's blood serum before and *D* after 16 injections of normal human serum albumin processed by E. R. Squibb & Sons by the "cold-alcohol" method and heated at 60°C for 10 hours. There were no changes in the patterns of the rabbit's serum after the injection of albumin. (Patterns *C* and *D* were scanned by Spinco Analytrol Model RB, which gives higher values than those obtained in *A* and *B*, scanned by Model RA.)

mic factor showed a considerable loss of activity, and the thrombocytopenic factor became inactive (Table 3).

#### Comment

The essential question concerning the effect of serum albumin on the marrow is

whether the phenomenon is a part of the hematopoietic mechanism. Two other possibilities, that of toxicity of a foreign serum and antigen-antibody reaction, were investigated and dismissed.<sup>3,6</sup> For the present, at least, it may be assumed that the action

TABLE 3.—Peripheral Blood Changes in Rabbit Following Twelve Intravenous Injections of Lyophilized Serum Albumin Stored in Vacuo for One Year—Blood Studies Forty-Eight Hours After Injection\*

Injections, No.	Hemoglobin Gm.	Erythrocytes Cu. Mm.	% %	Leukocytes Cu. Mm.	Differential Leukocyte Count		Thrombocytes Cu. Mm.	Reticulocytes, %
					Lymphocytes	Granulocytes		
Prior to injection	11.6	5.87	37	6,200	3,720	2,480	335,000	1.8
2	10.7	5.70	36	18,150	7,074	5,421	540,000	
3	10.1	5.81	35	11,900	5,950	5,831	460,000	
4	10.4	5.93	38	9,900	3,920	5,782	415,000	
5	10.4	5.84	35	10,200	4,968	5,103	325,000	7.1
6	10.4	5.56	35	9,100	5,278	3,640	350,000	
7	10.7	5.49	38	7,700	3,027	3,696	315,000	
8	10.4	5.60	35	9,000	6,030	2,970	370,000	4.4
9	10.7	5.69	36	10,200	8,670	2,326	455,000	
10	9.5	4.97	32	6,600	5,610	594	380,000	2.4
11	9.5	5.0	32	6,750	5,828	653	375,000	
12	8.6	4.41	32	4,050	3,720	250	475,000	1.4

\* Interpretation: Storage of lyophilized serum albumin for a period of one year destroys almost completely the anemia factor and the thrombocytopenia factor entirely but preserves the granulocytopenia factor.

on the marrow is exercised by one or more specific regulators which are contained in serum albumin. These regulators may be conceived to be elaborated somewhere in the body and delivered into the circulation, where they attach themselves to the albumin component. Another possibility is that the serum proteins have the function of hematopoietic regulation.

The author proposed the concept of the existence of a pair of circulatory hematopoietins for each cell type, which includes leukocytes, erythrocytes, and thrombocytes. One of the pair stimulates cell growth and maturation (hematopoeisin), and the other holds these properties to a physiological level (hematopenin)<sup>1</sup>. Since the hematopenins are the more potent of the pair of regulators, they produce significant changes which can be demonstrated. In accordance with this concept, these studies indicate that the hematopenins are associated with the albumin component of normal human serum.

The injection of various globulin fractions into the rabbits resulted in a granulocytic hyperplasia of the marrow and a slight leukocytosis of the peripheral blood. It is questionable whether the globulins may be conceived at the present phase of our investigations as carrying hematopoie-

sins. Cell hyperplasia has been limited to granulocytes and the degree was not consistent with that conceived for hematopoeisins.

That the regulators may be destroyed in the process of chemical separation has been demonstrated by the inactivity of the albumin prepared by the "cold-alcohol" method and by heating it at 60 C for 10 hours. It was shown in a previous publication<sup>3</sup> that heating of the whole serum at 60 C for 30 minutes inhibited all hematopoietic activity. However, there is evidence that when the fractions are separated not all the regulators are thermolabile. When the serum was fractionated with sodium tetrametaphosphate into several components, each with a specific activity, all but the anemia factor were found to be thermostable.<sup>4</sup> When the whole serum was preserved in a lyophilized form, it retained approximately 60% of its hematopoietic activity for most of the regulators even after 10 years. Lyophilized albumin after its separation from the serum proteins showed a decrease of activity after one year.

Paper-electrophoretic patterns of the animals given injections of hematopoietically active albumin obtained by continuous-flow electrophoresis showed a decrease of albumin and an increase in  $\beta$ - and  $\gamma$ -globulins.

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The increase in  $\gamma$ -globulins may be associated with multiple injections of an antigen. Those rabbits given injections of hematopoietically inactive Squibb albumin did not reveal any changes in the paper-electrophoretic pattern. The significantly lower percentage of albumin observed upon injection of hematopoietically active albumin is interesting from two points of view. The decrease in albumin in animals showing a suppression of marrow activity would support the concept of a relationship of albumin to hematopoietic regulation. Secondly, low albumin is frequently associated with leukemia,<sup>7</sup> a disease in which the hematopoietic mechanism is disturbed. That the decrease of albumin in the sera of the animals given injections of albumin is not a specific phenomenon, but denotes rather a general disturbance of hematopoiesis, is indicated by an equal decrease of albumin in animals given injections of various globulin fractions.

### Summary

Serum albumin obtained from normal human blood separated by continuous-flow electrophoresis and injected into rabbits produced marked inhibition of cell maturation and cell proliferation in the marrow. The peripheral blood reflected these changes by anemia, granulocytopenia, and thrombocytopenia. Albumin prepared by another method failed to act on the marrow. Paper-

electrophoretic patterns of the serum of those animals given injections of active albumin showed a consistent decrease of albumin.

The concept is advanced that the serum albumin is concerned with hematopoiesis. This protein constituent of serum contains regulators of hemocytopenin type, which control the degree of maturation and proliferation of cells in the marrow.

Toledo Hospital Institute of Medical Research, 2805 Oatis Ave. (6).

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# Mechanism of Hematopoiesis

## Hematopoietic Regulators in Serum Albumin

BERNHARD STEINBERG, M.D., ALBERT A. DIETZ, PH.D., and M. A. ATAMER, M.D., Toledo, Ohio

Investigations described in previous publications were interpreted to indicate that regulation of blood cells is associated in some manner with serum proteins.<sup>1-3</sup> Multiple intravenous injections of normal human serum<sup>1</sup> or of its albumin fraction<sup>3</sup> produced varying degrees of inhibition of production and maturation of cells in the marrow of rabbits. The cellular constituents of the peripheral blood reflected the changes in the marrow.

Further investigations attempted to determine the effect upon individual types of blood cells by specific fractions of serum proteins. If a fraction did exert control over a single type of blood cell and if a fraction effected an increase of one or more of the individual cells it would lend further proof to the concept that the hematopoietic mechanism is under regulation by the serum proteins. Of the several methods tried, fractionation with sodium tetrametaphosphate and ammonium sulfate was the most successful in achieving some of the aim (Fig. 1). A degree of specificity was obtained.<sup>2</sup> One fraction was found to be responsible for granulocytic hyperplasia in normal animals and a decrease in the proliferation of granulocytes in acute granulocytic leukemia. Another fraction suppressed the maturation of granulocytes, but increased the number of thrombocytes. A third fraction decreased the proliferation of erythrocytes and the maturation of granu-

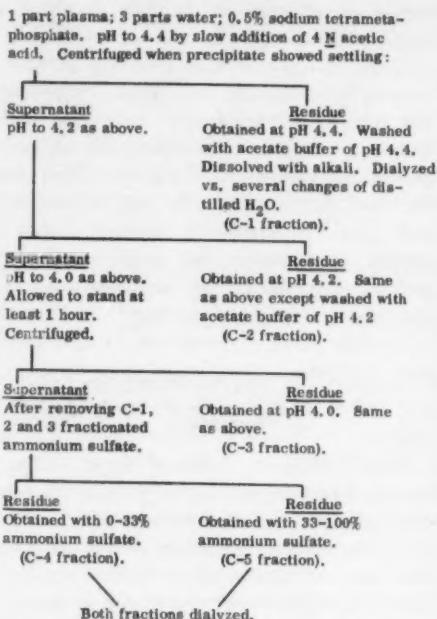


Fig. 1.—Fractionation of human plasma with sodium tetrametaphosphate and ammonium sulfate.

locytes (Tables 1 and 2). Although some specificity of action became apparent, the chemical procedures employed failed to accomplish complete separation. At the same time no fraction was chemically distinctive. Each one consisted of varying quantities of globulins and albumin (Table 3).

Since albumin was found to exert the hemocytopoietic effects of the whole serum,<sup>3</sup> the present study was undertaken to explore the effects upon hemocytopoiesis by the albumin components of the fractions obtained with sodium tetrametaphosphate, which had given an indication of specific regulation of individual blood cells. It was

Submitted for publication Oct. 20, 1958.

Toledo Hospital Institute of Medical Research.

This work was sponsored by a grant from the John A. Hartford Foundation, Inc.

Acknowledgment is made to Leon Libenson, Ph.D., for his aid in the continuous flow electrophoresis.

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TABLE 1.—*Hematologic Responses in Peripheral Blood After Injections of Sodium Tetrametaphosphate and Ammonium Sulfate Fractions of Normal Human Serum into Rabbits*

Fraction	Rabbits, No.	Peripheral Blood						Differential	
		Hemoglobin	RBC	WBC	Thrombocytes	Gran.	Lymph.		
C-1	34	No change	No change	No significant change	No change	Occasional slight increase	Occasional slight increase		
C-3&4	56	No significant change	No significant change	Equal to no. lymphocytes	Increased up to 2½ times	Reduced to 0 in most animals	No change or increase		
C-2&5	68	15%-50% decrease in most animals	10%-70% or more decrease	Equal to no. lymphocytes	Decreased in some animals by 50%-80% of normal	Reduced to 0 in most animals	No change or increase		

TABLE 2.—*Bone Marrow Response After Injections of Sodium Tetrametaphosphate and Ammonium Sulfate Fractions of Normal Human Serum into Rabbits*

Fraction	Rabbits, No.	Bone Marrow Changes in			
		Total Cellularity	Granulocyte Maturity	Erythroid Change	Megakaryocyte Change
C-1	34	Increased	Increased 45% to 100% over normal *	Increased * proliferation by 20%-40% over normal	Marked increase in no. by 300%-600% over normal *
C-3&4	56	Unchanged	Reduced or suppressed	Moderate increase by 60%-100% over normal	Slight increase by 20%-30% over normal
C-2&5	68	Usually unchanged	Markedly reduced or suppressed	Slight to moderate decrease of 40% ± 2%	No appreciable change

\* Marked decrease in number of granulocytes in acute granulocytic leukemia and a marked increase in megakaryocytes and a considerable increase in erythroid cells.

TABLE 3.—*Electrophoretic Components of Serum Fractions Obtained by Sodium Tetrametaphosphate and Ammonium Sulfate Precipitation*

Description of Fraction	Concentration, Mg. Nitrogen/Ml. Serum					Total Nitrogen
	α-	β-	γ-	Total Globulin	Globulin	
C-1 (removed at pH 4.4)	0.017- 0.039	0.067- 0.182	0.151- 0.168	0.080- 0.308 *	0.351- 0.886	
C-2 (removed at pH 4.2)	3.0- 3.82	0 (tailoring)	0 (tailoring)	0 (tailoring)	3.0- 3.82	
C-3 (removed at pH 4.0)	0.855- 1.61	0.228- 0.39	0.23- 0.244	0.17- 0.244	1.57- 2.4	
C-4 (33% am. sulfate)	0- 0.023	0.08- 0.115	0.35- 0.54	0.328- 0.68	0.80- 1.228	
C-5 (100% am. sulfate)	0.070- 0.100	0.082- 0.156	0.31- 0.486	0.83- 1.023	1.36- 1.735	

\* γ-Globulin is probably absent; may be inert material.

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also hoped that the study would add to the further chemical purification of the fractions, achieve a greater degree of biologic specificity and possibly identify other regulators.

### Experimental Procedures

New Zealand White rabbits of either sex and approximately 4 months of age were used in the experiments. All injections were given intravenously into the marginal veins. Multiple injections varying from 8 to 15 were given at two-day intervals. Each dose equalled 4.4 ml. of the original serum per pound of body weight of the animal. A total of 39 rabbits was used.

Blood for hematological and electrophoretic studies was obtained from the marginal veins prior to injections. Total leukocyte, erythrocyte, and thrombocyte counts were done with the use of a counting chamber, the last by the Brecher method. Hemoglobin was determined with the

electrophoretic colorimeter and the hematocrit by the micromethod. Reticulocyte counts and differential studies of the cells were made on stained slide preparations.

The serum proteins of normal human serum\* were fractionated with sodium tetrametaphosphate† and ammonium sulfate, as outlined in Fig. 1 and described elsewhere.<sup>2</sup> Five fractions were obtained. The effects produced by them in specific combinations were described previously<sup>3</sup> and are summarized in Tables 1 and 2. Each group of these fractions (1, 3&4, 2&5) were further fractionated by continuous flow electrophoresis into four subfractions. Electrophoretic Fraction CFE-1 consisted of  $\gamma$ -globulins; fraction CFE-2 was made up of  $\beta$ -globulins; CFE-3 contained  $\alpha$ -globulins and small amounts of albumin, and CFE-4 was composed of albumin with a trace of  $\alpha$ -globulins.

Each of the globulin and albumin subfractions obtained by continuous flow electrophoresis from Fractions 1, 3&4, and 2&5 prepared by sodium tetrametaphosphate and ammonium sulfate were injected into three to four rabbits from 8 to 16 times. As soon as changes in the peripheral blood became established, the animals were killed and the bone marrow and other organs were prepared for histological studies. Differential counts were made on 1,000 or more cells of the marrow. The maximum number of injections were given to those animals which did not reveal hematological abnormalities. Paper electrophoresis patterns were obtained on the serum of the animals during the course of the injections.

## Results

*Fractionation with Sodium Tetrametaphosphate.*—When Fraction C-1 (sodium tetrametaphosphate) of normal human serum was injected into normal rabbits there were no significant changes in the peripheral blood. The bone marrow, however, showed an increase in the proliferation of normoblasts and polymorphonuclear leukocytes. Especially marked was the increase in the number of megakaryocytes (Tables 1 and 2). Fraction C-1 was given to a patient with acute granulocytic leukemia with a leukocyte count which varied between

\* Serum was obtained from the hospital blood bank and from the American National Red Cross and its Toledo chapter by courtesy of Drs. Sam Gibson and Hoyt Meader.

† Obtained from the Victor Chemical Works, Chicago. The chemical is obtained under the trade name Cyclophos. Batches vary in precipitating serum proteins.

250,000 and 320,000 cells per cubic millimeter of blood, of which 99% were myeloblasts. Thrombocytes were 8,000. After the injection of C-1, leukocytes dropped below 10,000 (Fig. 2) and the bone marrow showed a marked reduction of myeloblasts with the appearance of erythroid cells and megakaryocytes (Fig. 3). However, neither erythrocytes nor thrombocytes were increased in the peripheral blood, nor was there evidence of significant maturation of granulocytes. The patient died of hemorrhage and bacteremia. This patient's case is presented to show the variable granulocyte response to the injection of C-1 in a normal animal and in a patient with acute granulocytic leukemia, to indicate that the stimulus to megakaryocytic proliferation was not followed by an increase of thrombocytes in the blood stream, and to present experimental proof that megakaryocytic hyperplasia and erythroid stimulation occur not only in the rabbit but also in man following administration of a specific fraction of normal human serum.

*Fractionation by Continuous-Flow Electrophoresis.*—Subfractionation of C-1 into four fractions was accomplished by continuous-flow electrophoresis. Subfraction CFE-4 contained 0.017 to 0.039 mg. of nitrogen per milliliter of original serum. The variation in concentration in various batches was due to varying quantities of albumin in the batches of sera and to the inadequacy of the method of separation. Subfraction CFE-4 was composed of albumin with a trace of  $\alpha$ -globulins. This subfraction was refractionated by continuous-flow electrophoresis into two components, "A" and "B." The "B" fraction contained approximately half of the albumin and was free of  $\alpha$ -globulin. When this fraction was injected into four rabbits it produced a more pronounced megakaryocytic hyperplasia and erythroid stimulation than was obtained with Fraction C-1. Another striking change was the increase in proliferation and maturation of granulocytes (Fig. 4B).

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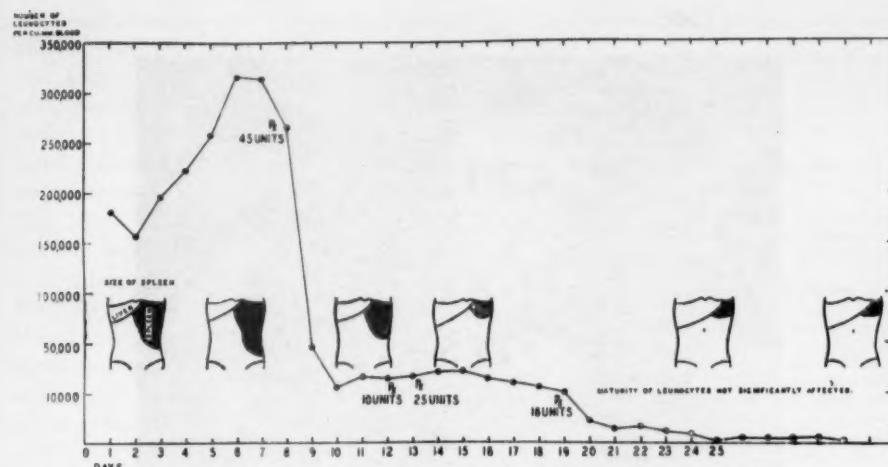


Fig. 2.—The graph shows the total leukocyte counts per cubic millimeter of blood and size of spleen in a patient with acute granulocytic leukemia. The patient received Fraction C-1 intravenously in amounts indicated by Rx. Each unit corresponds to 20 ml. of original serum. At time of treatment the total leukocyte count varied between 250,000 and 320,000. The leukocytes were 99% blast cells. After the injections were initiated the count dropped precipitously and the size of the spleen was reduced to within normal limits. These changes in the patient may be interpreted to indicate that C-1 contains regulators which stopped the proliferation of granulocytes.

Other subfractions of C-1 obtained by continuous flow electrophoresis contained  $\gamma$ -globulins (CFE-1),  $\beta$ -globulins (CFE-2) and  $\alpha$ -globulins with albumin (CFE-3). None of these fractions showed significant changes either in the peripheral blood or in the marrow when injected into nine rabbits.

**Fractionation with Sodium Tetrametaphosphate.**—When Fractions C-3&4 (sodium tetrametaphosphate and ammonium sulfate) were injected into rabbits, the

peripheral blood was found to contain an increased number of thrombocytes and absence of granulocytes (Table 1). The total leukocyte and erythroid counts remained within normal limits. The marrow showed a reduction or suppression of granulocytes and an increase of erythroid cells (Table 2).

**Subfractionation by Continuous-Flow Electrophoresis.**—Fractions C-3&4 were subfractionated by continuous-flow electrophoresis into four subfractions. CFE-4

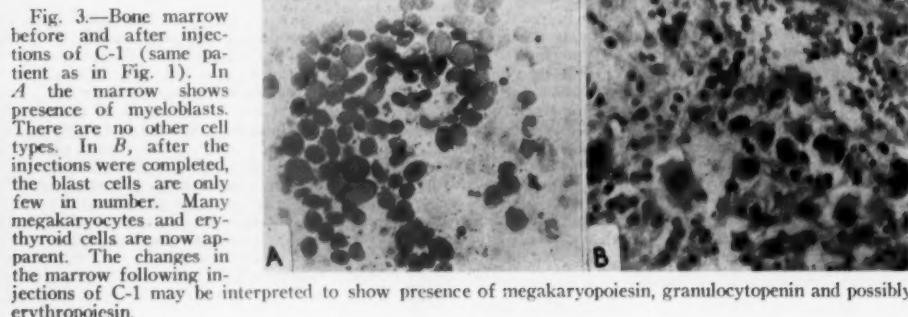


Fig. 3.—Bone marrow before and after injections of C-1 (same patient as in Fig. 1). In A the marrow shows presence of myeloblasts. There are no other cell types. In B, after the injections were completed, the blast cells are only few in number. Many megakaryocytes and erythroid cells are now apparent. The changes in the marrow following injections of C-1 may be interpreted to show presence of megakaryopoiesin, granulocytopoenin and possibly erythropoiesin.

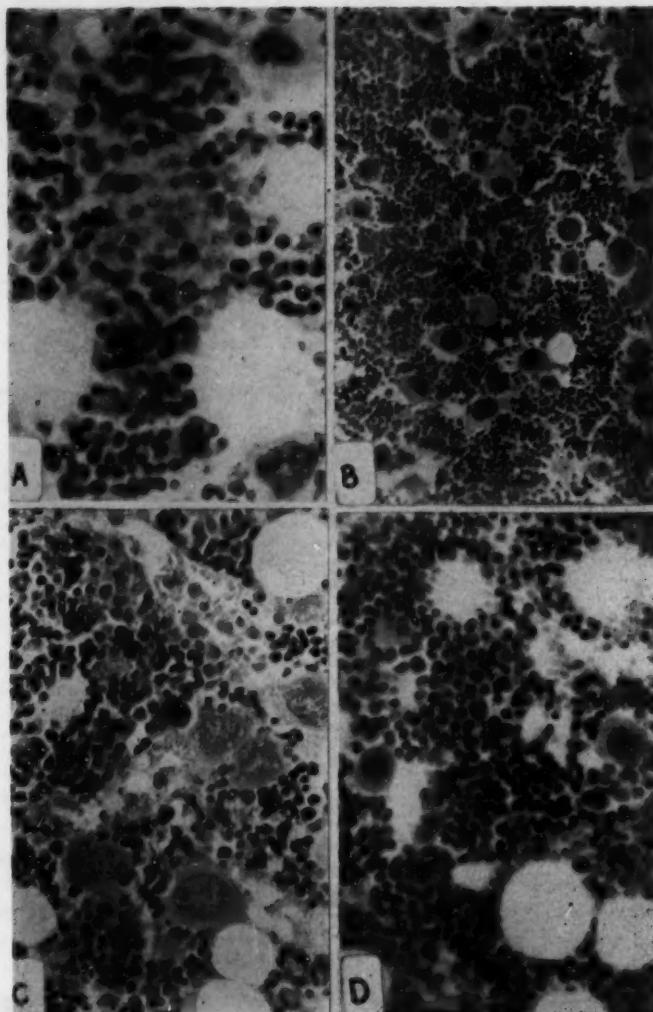


Fig. 4.—Photomicrographs of humeral marrow of rabbits, normal and after multiple injections of fractions obtained from normal human serum. Photomicrographs were taken at various magnifications (but within limits to allow comparison) to demonstrate the various cellular components. *A*, after CFE-4 (albumin) of C-3&4. No granulocytes are seen. There are two megakaryocytes and normoblasts in the field. The changes may be interpreted to indicate the action of granulocytopenin and erythropoiesin;  $\times 700$ . *B*, after CEF-4 (albumin) of C-1. Megakaryocytes are numerous. There is considerable increase in cellularity, contributed largely by polymorphonuclear leukocytes. The changes may be interpreted to indicate the action of megakaryopoiesin and granulocytopoiesis;  $\times 500$ . *C*, after CFE-4 (albumin) of C-2&5. There is an occasional granulocyte. Megakaryocytes are increased in number. There is some decrease in the number of normoblasts. The changes may be interpreted to indicate the action of megakaryopoiesin, granulocytopenin, and erythropoiesis;  $\times 800$ . *D*, normal rabbit marrow, showing distribution and number of megakaryocytes, granulocytes, and normoblasts. Compare with *A*, *B*, and *C*;  $\times 700$ .

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contained albumin with a trace of  $\alpha$ -globulins. When this subfraction was injected into four rabbits, the only change in the peripheral blood was a marked granulocytopenia. All other blood-cell elements remained normal, except for an increase in reticulocytes from 1.4% to 7.8% (Table 4). The bone marrow was cellular, with a marked decrease in the proliferation and maturation of granulocytes (Table 4). Normoblasts were increased 100%. A reticulocyte increase and a 100% greater number of marrow normoblasts over normal (Tables 4 and 6) suggests stimulation of erythropoiesis (Fig. 4A). It appears that Subfraction CFE-4 contains granulocytopenin and erythropoiesin.

TABLE 4.—Fractions C-3&4 Prepared with Sodium Tetrametaphosphate and Ammonium Sulfate and Further Fractionated by Continuous-Flow Electrophoresis into Four Fractions Which Were Injected Intravenously into Thirteen Rabbits Fourteen to Eighteen Times at Two-Day Intervals—Blood and Tissue Studies\*

Fraction No.	Electro- phoretic Composition	Changes in the Peripheral Blood	Changes in Marrow and Other Organs
CFE-1	$\gamma$ -Globulins	No changes	No changes
CFE-2	$\beta$ -Globulins	No changes	Increase in cellularity & maturation of granulocytes
CFE-3	$\alpha$ -Globulins & some albumin	No changes	Increase in cellularity of granulocytes with slight increase of granulocyte immaturity
CFE-4	Albumin & trace of $\alpha$ -globulins	Granulocytopenia from normal of 1,848 granulocytes to 35; reticulocytes increased from 1.4%-7.8%; no other changes	Marrow very cellular with marked decrease of proliferation and maturation of granulocytes (98.5%-97.9% of normal) & hyperplasia of erythroid cells (100% over normal) Fig. 4A

\* Interpretation: The albumin component of subfractions of normal human serum protein decreased the maturation and proliferation of marrow granulocytes. It is postulated that the albumin component obtained by continuous-flow electrophoresis from fraction processed by sodium tetrametaphosphate contains granulocytopenin, one of the regulators of hemocytogenesis. There is also suggestive evidence of presence of a regulator which increases proliferation and maturation of erythroid cells (erythropoiesin).

TABLE 5.—Fractions C-2&5 Prepared with Sodium Tetrametaphosphate and Ammonium Sulfate and Further Fractionated by Continuous-Flow Electrophoresis into Four Fractions Which Were Injected Intravenously into Thirteen Rabbits Eleven to Thirteen Times at Two-Day Intervals—Blood and Tissue Studies\*

Fraction No.	Electro- phoretic Composition	Changes in Peripheral Blood	Changes in Marrow & Other Organs
CFE-1	$\gamma$ -Globulins	Leukocytosis of 21,100; platelets from 380,000-945,000	Hyperplasia of granulocytes & erythroid cells in marrow; hypoplasia of splenic lymphoid tissue
CFE-2	$\beta$ -Globulins	Leukocytosis of 24,050; platelets from 340,000-870,000	Hyperplasia of marrow granulocytes & erythroid cells; lymphoid hypoplasia of spleen
CFE-3	$\alpha$ -Globulins & albumin	Hgb. from 11.9-7.7 gm.; RBC from 6,044-4,300; hematocrit from 40%-26%; slight leukopenia; granulocytes from 1,900-441; reticulocytes not affected	Marrow cellular but with suppression of granulocyte maturation and hyperplasia of megakaryocytes by 200%-400% over normal
CFE-4	Albumin with a trace of $\alpha$ -globulin	Granulocytopenia from 2,124-41; slight anemia (hgb. 8.9; RBC 4,18; hematocrit 30); reticulocytes from 3.7%-10.1%	Marrow very cellular but granulocyte maturation inhibited & markedly megakaryocytic increase of 400%-600% over normal (Fig. 4C)

\* Interpretation: The albumin component obtained by continuous-flow electrophoresis from the fraction processed by sodium tetrametaphosphate inhibits granulocyte maturation, increases production of megakaryocytes, and decreases the maturation of erythrocytes. It is postulated that the albumin fraction contains granulocytopenin, erythropoiesin, and megakaryopoiesin, which represent regulators of hemocytogenesis.

Subfractions of C-3&4, which included CFE-1, made up of  $\gamma$ -globulins; CFE-2, consisting of  $\beta$ -globulins, and CFE-3, composed of  $\alpha$ -globulins and albumin, were injected into nine rabbits. None of the animals showed any significant changes, either in the peripheral blood or in the bone marrow (Table 4).

*Fractionation with Sodium Tetrametaphosphate.*—When Fractions C-2&5 (Fig. 1) obtained with sodium tetrametaphosphate and ammonium sulfate were injected into rabbits they developed an anemia and

TABLE 6.—Differential Cell Counts of Bone Marrow from Rabbits Given Injections of Various Subfractions Obtained by Continuous Flow Electrophoresis from Fractions Separated by Sodium Tetrametaphosphate and Ammonium Sulfate from Normal Human Serum

Type of Cell	Normal	% of Each Cell Type (1,000 Cells Counted) of the Marrow of Rabbits							
		Material Injected *							
		CFF-1 of C-3&4	CFF-2 of C-3&4	CFF-3 of C-3&4	CFF-4 of C-3&4	CFF-1 of C-2&5	CFF-2 of C-2&5	CFF-3 of C-2&5	CFF-4 of C-2&5
Stem cell	0.	1.8	0.5	10.6	0.6	6.2	5.0	2.5	15.3
Myeloblast	1.0	0.	0.	0.	5.6	0.	0.	0.	0.
Promyelocyte	1.4	1.4	1.2	6.0	3.0	3.4	4.1	1.0	0.
Myelocyte	5.6	16.4	7.0	17.0	1.4	9.6	12.2	1.0	0.
Juvenile	13.2	14.2	13.0	21.6	0.8	16.2	20.4	1.0	0.
Polymorphonuclear	32.0	14.2	34.5	3.0	0.	10.4	21.0	0.	0.
Lymphocyte	6.2	0.4	8.5	4.0	2.8	6.6	9.0	12.0	25.0
Plasma cell	1.2	6.0	1.0	21.8	18.6	3.8	1.0	20.8	28.3
					(adult)				
					1.6				
					(young)				
Megakaryocyte	0.8	0.2	0.7	0.2	0.4	0.8	0.2	1.0	1.5
Young megakaryocyte	0.2	0.4	0.3	0.6	0.2	0.4	0.4	0.2	0.4
Pronormoblast	2.4	0.	0.	0.	1.6	0.	0.	0.	0.
Normoblast	33.8	39.0	33.0	34.2	66.0	42.6	26.8	38.4	29.5
					(no anemia)				
Monocyte	0.2	0.	0.	0.	0.4	0.	0.	0.	0.
									(anemia)

\* CFE indicates continuous flow electrophoresis, with numeral indicating number of the fraction; C, sodium tetrametaphosphate fraction, with numeral following C, such as C-1, indicating number of the fraction; CFE-1 contains  $\gamma$ -globulin; CFE-2 contains  $\beta$ -globulin; CFE-3 contains  $\alpha$ -globulin and some albumin; CFE-4 contains albumin with trace of  $\alpha$ -globulin.

granulocytopenia with a thrombocytopenia of 50% to 80% of normal (Table 1).<sup>2</sup> The bone marrow revealed marked reduction or complete suppression of granulocyte maturation and possible reduction of proliferation. There was a reduction of  $40\% \pm 2\%$  of normoblasts, due to a decrease of maturation, but no appreciable change in the number or appearance of megakaryocytes (Table 2).

*Subfractionation by Continuous-Flow Electrophoresis.*—Fractions C-2&5 were subfractionated by continuous-flow electrophoresis into four subfractions. CFE-4 contained albumin with a trace of  $\alpha$ -globulins. CFE-3 was composed of  $\alpha$ -globulins and albumin. When these subfractions were injected into seven rabbits, the peripheral blood showed granulocytopenia and anemia (Table 5). With CFE-3 the anemia was more marked and without change in the percentage of reticulocytes. With CFE-4, the anemia was slight but the reticulocytes were increased from 3.7% to 10.1%. Neither of the subfractions produced any change in thrombocyte counts. The bone

marrow showed presence of a very large number of megakaryocytes (400% to 600% above normal) with normal cytoplasmic content of thrombocytes. Maturation of granulocytes was suppressed at the level of stem cells (Tables 5 and 6). Other subfractions of C-2&5, which included CFE-1, made up of  $\gamma$ -globulins, and CFE-2, composed of  $\beta$ -globulins, produced some degree of leukocytosis and an increase of thrombocytes in the peripheral blood (Table 5).

On the basis of these observations, it appears that the albumin component of Fractions C-2&5 contains megakaryopoiesin, granulocytopenin, and erythropoienin. A trace of  $\alpha$ -globulins in CFE-4 suggests reservations for the present in the role of this component in hemocytogenesis.

#### Comment

These studies indicate that the albumin component of normal human serum is associated with a number of hemocytopoietic regulators which exert several different actions on the bone marrow of normal rabbits. There is suggestive evidence in our clinical

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investigations that human marrow is also affected by some of these regulators.<sup>2</sup>

An interesting and perhaps significant observation is the action of the regulator which produced hyperplasia of megakaryocytes without an increase of thrombocytes in the circulation (megakaryopoiesin). The cytoplasm of megakaryocytes was found to contain thrombocytes, indicating that the factor lacks the capacity to expel thrombocytes from the megakaryocytes into the blood stream. A similar condition may be seen in man and offers a perplexing clinical problem, especially when associated with thrombocytopenia. In a study to be published later, a thrombocyte-increasing factor (thrombopoiesin) will be described. This regulator does not produce a significant increase of megakaryocytes.

These observations suggest the presence of several distinct regulators which exercise control over the several phases of thrombocyte production. One regulator, megakaryopoiesin, is concerned with proliferation of megakaryocytes. Another regulator, thrombopoiesin, stimulates the production of thrombocytes. A third regulator may exist whose function is to expel thrombocytes into the blood stream.<sup>4</sup> Still another regulator holds down the production of thrombocytes (thrombocytopenin) as seen in the thrombocytopenia produced by the injection of serum albumin.<sup>3</sup> Our concept postulates that thrombocytopenin serves to hold the action of thrombopoiesin to physiological levels.

In the fractions obtained by methods described in this study, one and possibly two regulators for granulocytes have been identified. The regulator which reduced the proliferation and maturation of granulocytes (granulocytopenin) was found to be associated with the albumin components of Fractions C-2&5 and C-3&4. The globulin fractions, upon separation from the albumin, produced granulocytic hyperplasia and increased maturation in the marrow. Whether the globulins serve any function in the hematopoietic mechanism was not estab-

lished by these studies. The granulocytic hyperplasia was not considered significant.

The albumin subfraction of C-1 obtained by continuous-flow electrophoresis presented evidence of the existence of a regulator concerned with an increase in granulocyte proliferation and maturation in normal animals.

Regulators for erythrocytes were obtained from Subfractions CFE-3&4 of C-2&5. Subfraction 4 was composed of albumin with a trace of  $\alpha$ -globulins and Subfraction 3 contained  $\alpha$ -globulins and albumin. These subfractions decreased the production of erythroid cells by interfering with maturation (erythropenin). Subfraction CFE-4 of C-3&4 stimulated production of erythroid cells in the marrow (erythropoiesin). This subfraction, made up of albumin, increased normoblastic proliferation in the marrow but did not show an increase of erythrocytes in the circulation. It may be assumed that two regulators of erythropoietic activity have been identified. One of them appears to be concerned with proliferation and maturation of erythroid cells (erythropoiesin) and the other with the inhibition of these functions (erythropenin). The expulsion of erythrocytes into the blood stream may reside in another regulator as suggested in a previous article.<sup>4</sup>

The studies indicate that although no clear-cut chemical separation of hemocytopoietic regulators was achieved by the methods so far used, identification of several regulators of blood cells was made under the conditions of the experiments. It appears that the albumin component of normal human serum proteins is associated with hemocytopoietic regulation.

Whether the regulators represent hormones or hormone-like factors which are elaborated somewhere in the body and expelled into the blood stream where they become attached to the serum albumin or whether the serum proteins possess an intrinsic regulating effect on hematopoiesis has not been determined by these experiments.

TABLE 7.—Identification of Several Regulators of Hematopoiesis by Fractionation of Normal Human Serum by Chemical Procedures and Continuous-Flow Electrophoresis \*

Identity of Fractions	Hematopoietic Activities of Fractions	Suggested Names for Regulators
CFE-4 of C-1 (albumin)	Megakaryocytic hyperplasia of marrow Reduces proliferation of marrow granulocytes in acute granulocytic leukemia (?) Increases proliferation & maturation of granulocytes in normal animals	Megakaryopoesin Granulocytopenin (L) Granulocytopoiesin
CFE-4 of C-3&4 (albumin)	Reduces proliferation & maturation of marrow granulocytes Increases proliferation of erythroid cells	Granulocytopenin Erythropoiesin
CFE-3&4 of C-2&5 (albumin and α-globulin)	Megakaryocytic hyperplasia of marrow Decreases maturation of granulocytes Decreases proliferation of erythroid cells	Megakaryopoesin Granulocytopenin Erythropoienin

\* Interpretation: Several regulators of hematopoiesis can be identified in normal human serum. Most, if not all, of them are associated with serum albumin. Methods of separation employed in these studies merely indicate that several regulators are present. No chemical separation was achieved by the procedures.

### Summary

Fractions of serum albumin from normal people were obtained by action of sodium tetrametaphosphate and continuous-flow electrophoresis. The fractions produced several changes in the marrow and peripheral blood of rabbits. There is suggestive evidence that human marrow is also affected. The changes were interpreted to be caused by hemocytopoietic regulators associated with serum albumin. Several regulators of hemocytopoiesis were identified in the various fractions. Complete chemical separation of the regulators was not achieved by the procedures used thus far.

The concept that a pair of regulators exists for each cell type has been given further support by the above findings. The concept postulates that one regulator is concerned with cell proliferation and maturation. The other regulator of the pair holds these functions to a physiological level.

One of the regulators identified in the fractions of normal serum albumin stimulated proliferation of megakaryocytes (megakaryopoesin) without increasing the number of thrombocytes. This observation suggests the presence of more than one regulator in the proliferation of thrombocytes. It also offers an explanation of the clinical condition associated with thrombocytopenia without a significant change in marrow megakaryocytes.

Other regulators identified in the serum albumin were those which suppressed or stimulated proliferation and maturation of granulocytes (granulocytopenin and granulocytopoiesin) and erythroid cells (erythropoienin and erythropoiesin).

Toledo Hospital Institute of Medical Research, 2805 Oatis Ave. (6).

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# The Relation of the Pancreatic Ducts to the Islets of Langerhans

*Study of Three Cases*

**YONG H. YANG, M.D., and WARREN C. HUNTER, M.D., Portland, Ore.**

The pancreas is actually two glands in one, consisting of an endocrine and exocrine portion. The majority of workers are agreed that the endocrine portion, the islets of Langerhans, when fully formed has no connection with ducts, although many admit that they are formed in part from the duct system. The studies of many investigators on the embryology and regeneration of the pancreas of experimental animals have established that epithelial cells of the islets of Langerhans and acini alike are derived by differentiation from pancreatic duct epithelium. This ability of the duct epithelium to form other cells is certainly carried beyond the period of embryonic development and apparently persists throughout life. The ductules are connected both with the islets of Langerhans and with small mucous glands and occasionally with acini in the guinea pig.<sup>8,9</sup> Although connections between islets of Langerhans and ductal system in the human pancreas have been observed,<sup>6,7,10</sup> little is known about this subject in the human.

These observations throw a new light on the pathological study of the pancreas, since it is obvious that the condition found at autopsy may be either the end of a long process, tending always in one direction, or the summation of a longer or shorter series of regeneration. The relation of the pancreatic duct epithelium to the islets of Langerhans has been studied exclusively in experimental animals, but the question of the existence and frequency of direct con-

tinuity of islets of Langerhans with small ducts and formation of islets from ductal epithelium in the human pancreas beyond the embryologic period requires further study. When one considers the difficulty of demonstrating connections and relations between ducts and islets, at least by the study of serial sections, it seems possible that connections may exist unsuspected and unfound.

## Report of Cases

CASE 1.—Autopsy tissues, including the pancreas from an adult male, were received by mail, without any clinical history or other information. The tissue study disclosed marked hypertrophy and dilatation of the heart, coronary arteriosclerosis with myocardial fibrosis, arteriosclerosis of the aorta, and arteriosclerotic nephropathy. The pancreas was unremarkable grossly.

Microscopically, interesting and important features were found in the pancreatic sections. Islets of Langerhans in the better-preserved portions were unchanged, as were the secreting acini. Sections from one block revealed one small easily recognizable area of ductular epithelium, surrounded by adult connective tissue, and attracted attention because of the cellularity and dissimilarity to normal duct parenchyma or islets of Langerhans (Fig. 1). At first glance this seemed to be an alveolar-forming structure, and such it was in part, but much of it, when examined more closely, had a little connective tissue and capillary vessels at the center of masses, indicating a papillary and irregular cord pattern and resemblance of islet cells (Fig. 2). The striking feature was the epithelium, since the cells were definitely different from the normal acini or islet cells.

Submitted for publication Sept. 30, 1958.  
From the Department of Pathology, University of Oregon Medical School.

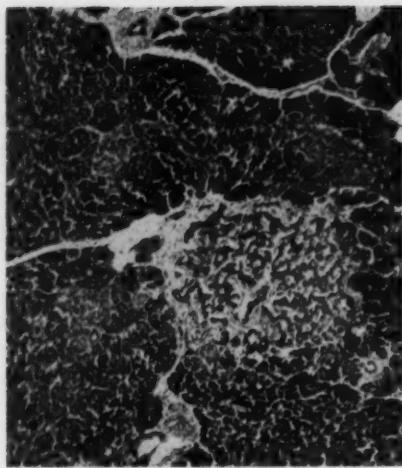


Fig. 1 (Case 1).—An interlobular pancreatic duct shows unusual hyperplasia of epithelium arranged in irregular cords with surrounding connective tissue. There is a direct continuity of the lesion with other known small ducts. Hematoxylin and eosin;  $\times 48$ .

However, they were thought to be undifferentiated ductal epithelium for the following reasons:

1. The columnar cells had pale homogeneous cytoplasm lacking zymogen granules.
2. The lesion was surrounded by collagenous connective tissue, well demonstrated by the Van Gieson stain.
3. A Gomori aldehyde fuchsin stain lacked dark blue  $\beta$ -granules, although the

islets of Langerhans elsewhere gave a positive reaction (Fig. 3).

4. In a series of 49 step-sections, made from the same block and cut to the point of exhaustion, there was established the continuity of the somewhat papillary focus as well as surrounding connective tissue with a known small duct nearby.

This lesion appeared to be the proliferation of ductal epithelium, transforming into islet cells where flat columnar and cuboidal cells were irregularly anastomosed in cords, separated by many capillaries with which the cells were in intimate connection.

CASE 2.—This patient was a 57-year-old white man who had suffered from persistent cough, with one-half to one cup of white mucoid sputum per day, and dull anterior chest pain, especially on the right side, for four months. He had lost about 40 lb. of weight since the onset of his illness. Chest x-ray revealed a large right hilar mass, with partial atelectasis of the middle lobe of the right lung. The left humerus, scapula, and fourth anterior rib revealed osteolytic densities by x-ray. A biopsy specimen of left humerus was reported as anaplastic carcinoma. Palliative x-ray therapy to the left shoulder had been completed with total 1,500 r. Autopsy showed anaplastic bronchogenic carcinoma of the right lung, with metastases to liver, various lymph nodes, bones (ribs, left humerus, scapula, and lumbar vertebrae), duodenum, transverse colon, both pleurae, right adrenal gland, and pancreas. There was no indication of any form of endocrine disorder clinically. The cause of death was a chronic active peptic ulcer of duodenum with recent intestinal hemorrhage.

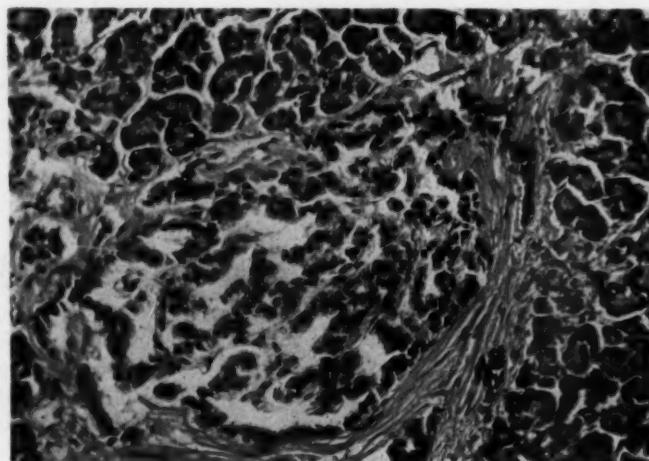
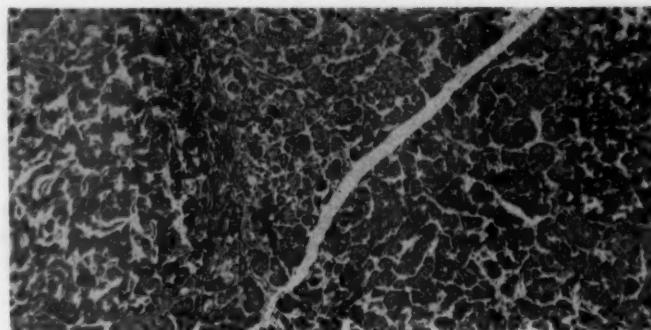


Fig. 2 (Case 1).—Higher magnification of same area discloses lower columnar cells uniform in size, shape, and staining reaction. Many capillaries are evident. Hematoxylin and eosin; reduced 35% from mag.  $\times 240$ .

## PANCREATIC DUCTS AND ISLETS OF LANGERHANS

Fig. 3 (Case 1).—Gomori aldehyde fuchsin stain shows no  $\beta$ -cell granules in the lesion, although the fully developed islet of Langerhans (right) gives a definite positive reaction. Hematoxylin and eosin; reduced 35% from mag.  $\times 95$ .



estimated at about 600 cc. Grossly the pancreas was firm and of normal size and consistency. The cut surface showed no evidence of cysts, hemorrhage, or fibrosis. Peripancreatic lymph nodes were not enlarged or involved by neoplasm. A careful examination revealed no abnormality of the main pancreatic duct.

Microscopically, there was a small area of anaplastic carcinoma in the block cut from the body of pancreas, which was felt to be a metastatic lesion from the right bronchogenic carcinoma. Of paramount interest were altogether very few islets of Langerhans situated near small pancreatic ducts, which disclosed one or more hollow structures, consisting of duct epithelium and lying definitely within the islets of Langerhans (Figs. 4 and 5). There was also evidence of direct extension of ducts into some islets. Five more blocks were

taken from different areas of the head, body, and tail of the pancreas, and all revealed many islets of Langerhans very closely approximating interlobular and intralobular small pancreatic ducts. Many serial step-sections of these blocks disclosed many islets of Langerhans, with inclusion of one or more hollow structures of duct-like epithelium, as well as definite evidence of direct extension of ductules into the islets of Langerhans. The inclusion bodies of hollow form within the islets of Langerhans in no way resembled the poorly differentiated cells of the metastatic carcinomatous focus in the pancreas. There was one area, demonstrating an islet of Langerhans surrounded by hyperplastic small ducts and connective tissue, in which there was not only direct continuity of ducts

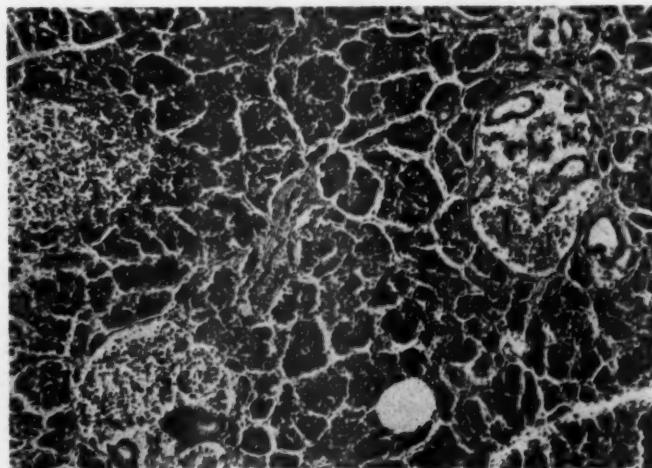


Fig. 4 (Case 2).—Many of ducts are nearby islets of Langerhans, and ductal structures are seen inside of islets. Hematoxylin and eosin; reduced 35% from mag.  $\times 95$ .



Fig. 5 (Case 2).—Higher magnification of right upper corner field of Figure 4 reveals the hollow appearances of undifferentiated ductal epithelium and fragmented aggregation of small islet cells. Hematoxylin and eosin; reduced 10% from mag.  $\times 400$ .

with islets of Langerhans but also transformation of duct epithelium into islet cells.

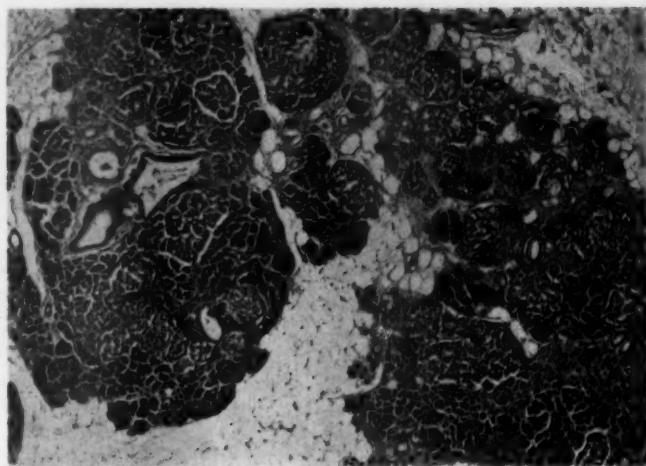
**CASE 3.**—A 78-year-old white man was hospitalized at Multnomah County Hospital from Nov. 25, 1957, to Dec. 6, 1957. His chief complaints were a 20-lb. weight loss in the last four months and productive cough for the past six months. Chest x-ray revealed evidence of neoplasia in the right pulmonary hilum and right lower lung field. Bronchoscopy and scalene lymph node biopsy were negative for malignancy. Because of x-ray appearance and patient's age and condition it was thought that a thoracotomy was not justifiable. The second admission was April 12, 1958, with chief complaints of ankle edema, productive cough, 30-lb. weight loss, and postprandial emesis on three occasions. The patient was treated for cardiac failure and ankle edema which responded quickly.

Chest x-ray disclosed constant evidence of neoplasia of the right hilar region and the lower lobe of the right lung. A diagnosis of bronchogenic carcinoma was made. Other findings were generalized arteriosclerosis, benign hyperplasia of prostate, and deafness. Several days prior to his death, pulmonary atelectasis and bronchopneumonia of right middle and lower lobes and extensive pleural reaction over the right side were noticed by radiographic examination. BUN was 24; hemoglobin, 13.4 gm.; white blood cell count, 15,000, with segmented neutrophils, 76; band cells, 3; lymphocytes, 17; monocytes, 1, and disintegrated cells, 3.

The body was well developed but extremely emaciated and weighed approximately 130 lb. Important autopsy findings were acute bronchopneumonia with multiple abscess formation, bi-

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Fig. 6 (Case 3).—Hyperplasia of small ducts and islets of Langerhans are shown. Hematoxylin and eosin; reduced 40% from mag.  $\times 70$ .



lateral; acute fibrinous pleuritis, right; pulmonary emphysema, bilateral; anaplastic bronchogenic carcinoma of right lower lobe, with metastases to right pulmonary hilar lymph nodes and right adrenal gland; hypertrophy of left ventricle of heart; generalized arteriosclerosis, and hyperplasia of prostate.

The pancreas was grossly unchanged and showed no evidence of neoplasm either grossly or on microscopic examination. No gross abnormality of main pancreatic ducts was noted. Histologically, diffuse hyperplasia and hypertrophy of islets of Langerhans and proliferation of small pancreatic ducts were marked (Fig. 6). Many small ducts were evident nearby islets of Lang-

erhans. Interesting findings were the direct continuity of secreting ducts and islets of Langerhans. Two small hollow structures of duct epithelium which were definitely different from islet cells, as evidenced by their densely stained nuclei and flat columnar shape, were present within one of islets. Other focal areas disclosed an entrance of a small duct in the islet without any intervening connective tissue septum (Fig. 7) and positive evidence of ductal epithelium in an islet and entering into the larger duct (Fig. 8). There was also suggestive transformation of islet cells from differentiating duct epithelium. These duct cells were very

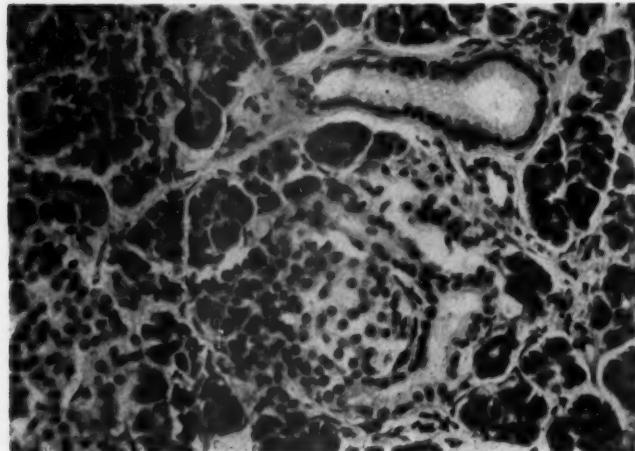


Fig. 7 (Case 3).—A small duct directly alongside the islet is demonstrated. Hematoxylin and eosin; reduced 40% from mag.  $\times 400$ .

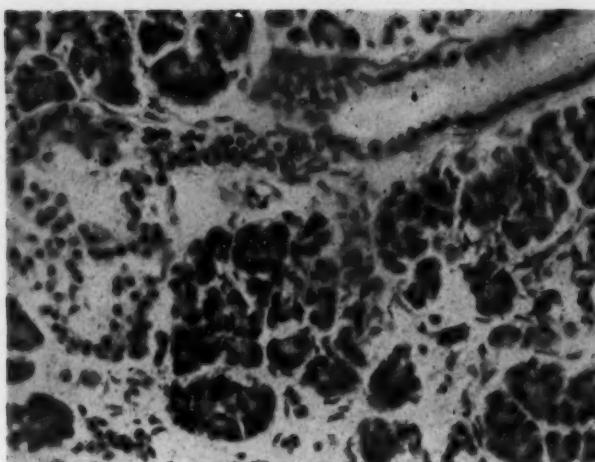


Fig. 8 (Case 3).—Direct connection of larger duct and islet of Langerhans by means of solid group of cells is well shown. Hematoxylin and eosin; reduced 40% from mag.  $\times 500$ .

similar in their staining reaction, shape, and arrangement to islet cells.

#### Comment

The relation of the islets of Langerhans to the ducts and acini of the pancreas has been the subject of considerable interest. Our Case 1 demonstrated a focus of unusual proliferation of pancreatic duct epithelium having an arrangement similar to islets of Langerhans overlying a capillary network; the individual epithelial cells lacked any granules in their cytoplasm and were small columnar and cuboidal cells with nuclei paler than normal duct epithelium. Serial sections of the pancreas of Cases 2 and 3 revealed quite a few well-formed ducts within the islets of Langerhans as well as direct continuity of minor pancreatic ducts with islets. These cases offer evidence of transformation of ducts to islet cells by means of proliferation of duct epithelium. The cases further demonstrate direct continuity of islet tissue with ducts and establish the histogenesis of islets of Langerhans from ductal epithelium in the adult human.

Laguesse<sup>1,2</sup> has demonstrated the continuity of islet and acinus tissue as well as the connection between small ducts and islets, supporting his descriptions by drawings of serial sections which show indubitably

table direct contact of islet cells and acinus cells without any intervening connective tissue septum. At an early embryonic period in the sheep, Laguesse observed the primitive gland structure of the pancreas is present here and there, and these cells proliferate and form protruding loops. They remain in connection with the tubules for some time and constitute the "primary islands." Later these structures atrophy and are replaced by similar bodies formed by proliferation of the fully formed secreting tubules and eventually become tunnelled and vascularized. He believed that it is possible for these structures to revert to the glandular type. However, later he modified his concept that some of the islands persist as such throughout life. On the other hand, Diamare<sup>3</sup> insisted that the islands are constant and unchanging formations, formed early in embryonic life and persisting until death, and that no transformation occurred from acini to islets of Langerhans. Von Hansemann<sup>4</sup> (1901) stated that the islands of Langerhans originate in late embryonic life by a proliferation of the connective tissue cells of the stroma. In 1903, Pearce<sup>5</sup> studied the histogenesis of islands of Langerhans of the human embryo and concluded that these originate through a proliferation and differentiation of the cells of the primitive

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secreting tubules. He observed that the differentiated cells, characterized by a rich, finely granular, eosinophilic protoplasm, lie as small round or oval masses in direct continuity with the cells of the tubules. Later, in an embryo of about the third month, a few entirely isolated islands were seen. Helly<sup>6</sup> (1906) and Weichselbaum and Kyrle<sup>7</sup> (1909) also observed islets in connection with ducts in the human pancreas; however they did not express clearly their views as to the frequency of this connection in the adult gland. On the other hand, there are many authors who not only deny any connection of the islets with the ducts but even maintain that those located in the lobules of the pancreas are wholly independent of the surrounding acinus tissue. By means of a combined staining method of the pancreas, Bensley<sup>8,9</sup> has been able to show that a system of anastomosing small ductules, having a diameter of  $12\mu$  to  $27\mu$ , which arise from the large ducts are connected with both the islets of Langerhans and acini in the guinea pig. He also expressed the opinion that normal regulation of islet content in the pancreas is by interstitial growth of preexisting islet and by the formation of new islets from the duct epithelium and not at all by the formation of new islets out of acini. In 1924, Nakamura<sup>10</sup> observed a well-developed island of Langerhans formed inside of a secretory duct with attachment to the same duct in the pancreas of a child, while studying 90 cases of pancreas. Hard<sup>11</sup> (1944) observed that the islets originate from three and possibly four sources in the embryonic and early postnatal stages of development of the rat. The first islets differentiate in the 13-day embryo from the wall of the solid pancreatic cord. The majority of embryonic islets take their origin from the pancreatic tubules. During the first week of postnatal life there occurs a considerable increase in the formation of new islets, and these arise from two sources. The majority originate from terminal portions of the secretory duct system at the base of acini. A lesser number of islets are

developed from the larger ducts, similar to those formed in embryonic periods from the pancreatic tubules. Bencosme<sup>12</sup> (1955) has distinguished between primary and secondary islets and summarized his study on histogenesis of pancreatic islets of rabbits. He stated the primary islets are derived from the primitive pancreatic cords and secondary islets appear after the formation of acini in rabbit embryos. He observed newly formed islet cells derived from the duct system, mostly from the centroacinar cells, as well as from the mitosis of immature islet cells preexisting in the wall of the acini.

Many experiments and observations demonstrate that the pancreas is capable of regeneration both in experimental animals and in pathological and physiologic states, as displayed by growth of acinar and islet cells with evidence of proliferation of the ducts and ductules. There occurs, to a limited extent, a proliferation of the pancreatic ducts and acinar cells and the appearance of new islands of Langerhans after partial excision of pancreas. If, however, obstruction of the pancreatic ducts occurs, the ducts dilate, and there is at first acinar atrophy, followed by slower disintegration of the original islands; but, at the same time, ducts begin to proliferate and give rise to many new islands and to some new acini. Kyrle<sup>13</sup> (1908) studied the reaction of the pancreas after partial removal and after portions of the gland were transplanted into spleen. He described an active proliferation of the duct epithelium, resulting in the formation of branching adenomatous structures, some of which later developed an acinus-like appearance and formed zymogen granules. Several islet buds originating from separate ducts may become confluent, to form a single islet. According to Bensley,<sup>8,9</sup> there are regenerative efforts of the pancreas of the rabbit and guinea pig after ligation of its ducts. He mentioned regenerative processes resulting in the formation of new acini and new islets from the remains of the duct system. Later, Grauer<sup>14</sup> ligated rabbit pancreatic

duct near the bowel, and his experiments and observations showed regenerative evidence of ducts similar to Bensley's experiments. Barron<sup>18</sup> (1920) observed the formation of young slightly irregular acini from the hyperplastic ducts in a case of pancreatic lithiasis, although he did not suggest any attempts at regeneration of the islets. Allen<sup>19</sup> (1922) reported distinct hypertrophy of the remaining tissue in five cases of a large series of pancreatectomized dogs. In one of these he mentioned evidence of proliferation of the ducts. Fisher<sup>17,18</sup> (1924) removed all of the pancreas from young dogs, except a portion of the tail, and stripped the glandular tissue from the main duct for a length of 2 in. from the duodenum. One of the dogs showed regeneration of a piece of pancreatic tissue from the segment of duct inside of the duodenal wall. In the studies of diabetic pancreas, there is clear evidence of regeneration of islets of Langerhans, although the degenerative changes predominate (Weichselbaum,<sup>19</sup> Boyd and Robinson,<sup>20</sup> and Warren and Root<sup>21</sup>).

The islets respond to various stimuli, either pathologic, physiologic, or experimental. Sergeyeva<sup>22</sup> observed an increase in the numbers of  $\alpha$ -cells after adrenergic stimuli and an increase in the numbers of  $\beta$ -cells after cholinergic stimuli. The acute effect of insulin, as reported by Kogan,<sup>23</sup> consists of an increase in the number and size of the islets. Reports on the results of prolonged administration of insulin are rather contradictory, probably because of the widely varying dosages used by different workers. Many (Herring<sup>24</sup> and others) have reported hypertrophy and hyperplasia of the islets; however, proliferative activity of the islets is found inhibited (McJunkin and Roberts<sup>25</sup>) and reduced (Evans and Haist<sup>26</sup>) after continued administration of insulin. On the other hand, Schmid<sup>27</sup> saw no changes in the amount of islet tissue. Pregnancy is accompanied by hyperplastic changes in the islets, according to Rosenloecher<sup>28</sup> and Florentin and Picard,<sup>29</sup> but Allen<sup>30</sup> failed to notice any change

in the pancreas of pregnant bitches. According to Sommers, Murphy, and Warren,<sup>31</sup> papillary or adenomatous hyperplasia of pancreatic ducts was associated with 41% of their 141 cases of pancreatic carcinoma seen at autopsy. Postmortem studies of 150 cases without cancer or clinical pancreatic disease revealed only a 9% incidence of papillary hyperplasia of pancreatic ducts, while comparable hyperplastic changes of duct epithelium were found in 28% of a separate series of 100 cases with diabetes mellitus. The incidence of pancreatic duct hyperplasia with rectosigmoid carcinoma in males is usually high. Neoplastic disease frequently involves the endocrine-stimulated organs, and the high incidence of pancreatic duct hyperplasia with carcinoma and diabetes mellitus is probably caused by a generalized endocrine imbalance and may be related to stimulation of islet cell regeneration. Starvation seems to cause a considerable increase in the amount of islet tissue (Vincent and Thompson,<sup>32</sup> Fischer,<sup>33</sup> Vincent,<sup>34</sup> and Stefko<sup>35</sup>). Chiovenda<sup>36</sup> found a marked increase in the amount of islet substance in cases of cachexia due to cancer and lymphogranulomatosis. Our Cases 2 and 3, whose glands revealed many foci of regeneration of islets, were poorly nourished and extremely cachectic owing to bronchogenic carcinoma with multiple metastases.

It is apparent from reviewing the literature that the islets of Langerhans are derived from differentiation of the duct system embryologically and, furthermore, that islets are able to regenerate from ductal proliferation and differentiation when necessity demands, notably after pancreatic injury for a variety of reasons. However, quantitative data as to occurrence under normal and under pathological conditions are still obscure.

We are assured that there is direct continuity of some islets of Langerhans with either small ducts or acini or both and that the close relationship as to transformation of islet cells from ductular epithelium exists beyond the embryological state. Islet cells

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are capable of further growth or regeneration by new formation from ducts, aside from the division of their own cells. How many islets have direct continuity with ductules in the fully developed human pancreas, how far this regenerative effort goes in restoring the organ to physiologic competency, and by what kind of stimuli are interesting and important questions. Even if there is some connection between islets and ductal system, these ducts are not necessarily discharging substances produced by the islets. We believe that while the connection of islets with duct system is present in regenerative states of islet cells, normally, once the islets of Langerhans are fully formed, there is no longer the continuity with ducts.

### Summary

Examples of proliferation of duct epithelium, transforming into islet cells, and direct continuity of some of islets of Langerhans with small ducts in the human adult pancreas are presented. The close relation between pancreatic duct and islets of Langerhans from the viewpoints of histogenesis and regeneration of islet cells are discussed.

Department of Pathology, University of Oregon Medical School, 3181 S. W. Sam Jackson Park Rd. (1) (Dr. Hunter).

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# Pathologic Studies in Eosinophilic Lung (Tropical Eosinophilia)

## *Case Reports*

T. J. DANARAJ, M.D., M.R.C.P.E., Singapore

Of the various conditions associated with an eosinophilia, there is a single clinical entity, called eosinophilic lung or tropical eosinophilia, characterized by persistent hypereosinophilia and chest symptoms responding favorably to treatment with organic arsenicals. Since the early reports by Frimodt-Möller and Barton<sup>1</sup> and Weingarten,<sup>2</sup> numerous others<sup>3-12</sup> have appeared confirming the existence of this condition in tropical and semitropical countries, but its etiology has remained obscure. More recently, however, the favorable response to treatment with diethylcarbamazine<sup>13-16</sup> and the finding of positive serologic reactions to the filarial complement-fixation test<sup>17,18</sup> have suggested that the condition may be due to some form of filarial infection, possibly involving species of filarial worms normally found in nonhuman hosts. It can not yet be assumed that this disease is a form of filariasis, but these newer findings have made it possible to define further eosinophilic lung and set it apart from other eosinophilias of obscure causes. The features by which it is now differentiated are (*a*) eosinophilia of 3,000 or more cells per cubic millimeter; (*b*) cough and shortness of breath, often severe; (*c*) pulmonary shadows in the roentgenograms; (*d*) increased erythrocyte sedimentation rate; (*e*) positive filarial complement-fixation test (FCFT) with titers usually above 1:10, frequently 1:80 or higher, and (*f*) prompt relief of symptoms and decrease in

FCF titer and eosinophilia following the administration of either organic arsenicals or diethylcarbamazine.

Knowledge of the underlying pathologic changes in eosinophilic lung has been limited because the condition is benign, any fatalities that have occurred being due to encephalopathy resulting from treatment with organic arsenicals. The purpose of this paper is to report such a fatal case which came to necropsy. In addition, the histologic changes seen in liver and lymph node tissue obtained at biopsy from patients suffering from eosinophilic lung will be described.

## **Report of Case**

A Ceylonese man aged 32 years was admitted to the General Hospital, Singapore, under my care, on Jan. 13, 1957, presenting the clinical picture of encephalopathy following treatment of eosinophilic lung with neoarsphenamine injections; he died 24 hours later.

He was a recent immigrant from Ceylon, having arrived in early 1956, and was very well till November of that year, when he developed cough which was diagnosed as tracheitis by the family doctor and treated symptomatically. The cough, however, persisted, and toward the end of December it was associated with breathlessness. When examined on Dec. 31, he was found to have abnormal physical signs suggestive of bronchial asthma. A roentgenogram of the chest showed accentuation of lung markings and soft mottled shadows which, although present throughout both lung fields, were most numerous in the basal and mid-zone areas. A differential leukocyte count showed an eosinophilia of 42%; a total leukocyte count was not done. Two years previously, while in Ceylon, he had suffered from a similar complaint, which was diagnosed as "tropical eosinophilia" and cured with acetarsone (Stovarsol) tablets. In view of the asthma-like symptoms, the radiologic

Submitted for publication Oct. 10, 1958.

From the Department of Medicine, University of Malaya.

picture, and hypereosinophilia, he was considered to be suffering from a second attack of the same condition diagnosed in Ceylon. He was, therefore, treated with a course of five injections of neoarsphenamine, the first being given on Jan. 5, 1957, and the remainder daily from Jan. 7-10. There was considerable improvement, and on examination no bronchial spasm was evident. When seen on the morning of Jan. 13, he complained of headache and slight fever, and by the evening had become unconscious. He was then admitted to the hospital.

**Physical Examination.**—Physical examination revealed an obese patient who was afebrile and in deep coma. The heart sounds were normal; crepitations were heard over both lung bases, and the spleen was palpable 2 fingerbreadths below the costal margin; the liver was not palpable. There was no obvious paralysis, but all the limbs were flaccid, with absent tendon reflexes; the plantar responses were extensor.

**Laboratory Findings.**—The cerebrospinal fluid was clear and under increased pressure, with 30 cells (lymphocytes) per cubic millimeter, 250 mg. of protein, 730 mg. of chloride, and 43 mg. of sugar per 100 ml.; no organisms were seen on stained smears. The blood leukocyte count was 31,000 cells per cubic millimeter, with polymorphonuclears 81%, lymphocytes 15%, mononuclears 1%, and eosinophils 3%.

He was treated with frequent injections of dimercaprol but died the next day without regaining consciousness.

**Necropsy Observation:**—The body was that of an obese man. The pericardial sac contained approximately 50 cc. of slightly blood-stained fluid. The heart was not enlarged, and the valves were competent. The trachea and bronchi contained dark fluid blood and froth. No enlarged hilar or mediastinal lymph nodes were found. The posterior surfaces of both lungs showed extensive subpleural hemorrhages and their cut surfaces poured dark edema fluid, but there were no areas of consolidation or cavitation. The capsule of the liver was smooth, and its cut surface was pale and fatty. The spleen was enlarged to about twice the normal size, and its pulp was red. The cut surfaces of the kidneys showed some congestion. No abnormality was found in the gastrointestinal tract, and the mesenteric lymph nodes were not enlarged. The leptomeningeal vessels were congested; multiple coronal sections through the brain revealed no abnormality except for a translucent appearance suggestive of cerebral edema.

### Microscopic Findings

**Lungs.**—Serial sections were made from several blocks of pulmonary tissue obtained from each lobe of both lungs and stained

with hematoxylin and eosin, Giemsa's stain, and by periodic acid-Schiff procedure (PAS). The histologic features, which were essentially similar throughout, consisted of scattered pneumonic areas with frequent focal granulomatous lesions. The alveolar septa in the affected areas were thickened and congested, and the alveoli were filled with an exudate consisting mainly of eosinophil and neutrophil polymorphonuclear leukocytes and large macrophages laden with brownish-golden pigment; a few lymphocytes and plasma cells were also present. In some of the exudative areas, focal granulomas of varying sizes, 0.2-0.8 mm. in diameter, were seen. The central portion of the larger granulomatous lesions consisted of very large multinucleated giant cells of variable sizes and shapes, each in most cases containing 20-30 nuclei gathered together in the center of the cell (Figs. 1-6). Among the giant cells, and occasionally within them, was found an eosinophilic, structureless, necrotic substance, which was rather compact and more refractile than the adjacent tissue from which it was clearly distinguished. This necrotic material, which was PAS-positive and not birefringent, occurred in irregular masses with frayed margins and frequently as thin twisted fibers (Fig. 2). In the periphery of the granulomas were leukocytes, mainly lymphocytes, plasma cells, and large pigment-laden macrophages. The smaller granulomatous lesions did not contain any necrotic material but only a few giant cells centrally placed surrounded by leukocytes. The granulomas were avascular and not found near blood vessels or bronchioles. There was no evidence of inflammation of the bronchi and bronchioles, and their lumina did not contain any exudate. The walls of the arteries and arterioles showed no abnormality.

**Liver.**—The liver cells, especially those around the central vein, showed a marked degree of fatty degeneration. The bile ducts were normal, but the periportal areas were infiltrated by large numbers of lymphocytes. No granulomas were seen in serial sections of several blocks of liver tissue.

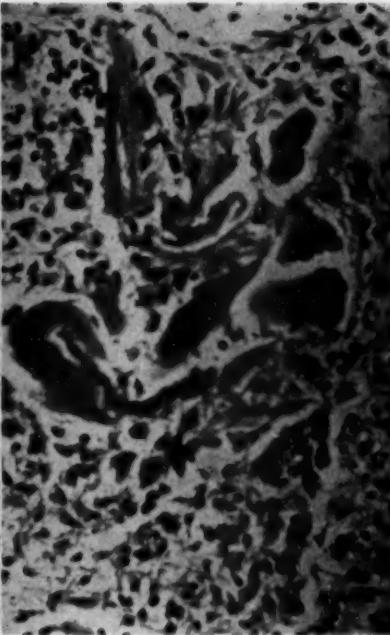
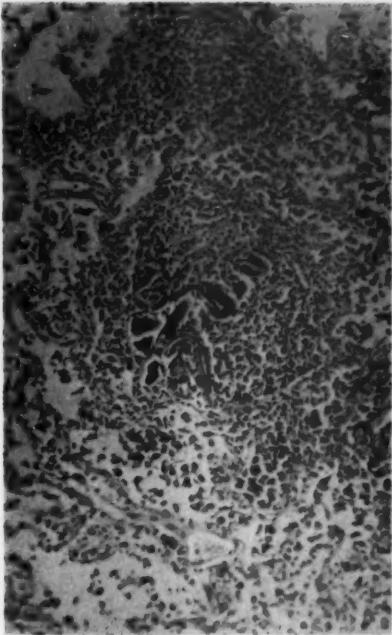


Figure 2

Figs. 1-6.—Photomicrographs illustrating granulomatous lesions in the lungs. Central area of foreign-body giant cells and necrotic material surrounded by leukocytes, mainly lymphocytes, plasma cells, and large pigment-laden macrophages. Necrotic material was not evident in the smaller lesions. Hematoxylin and eosin stain. Each figure: upper  $\times 100$ ; lower  $\times 330$ .

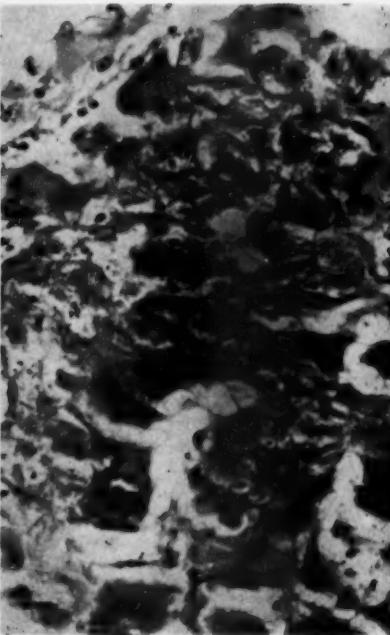
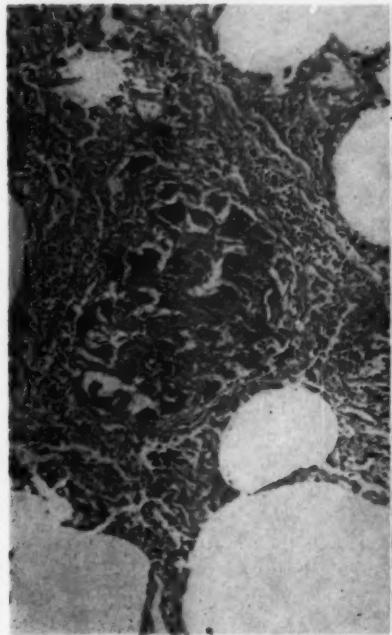


Figure 1

Fig. 4

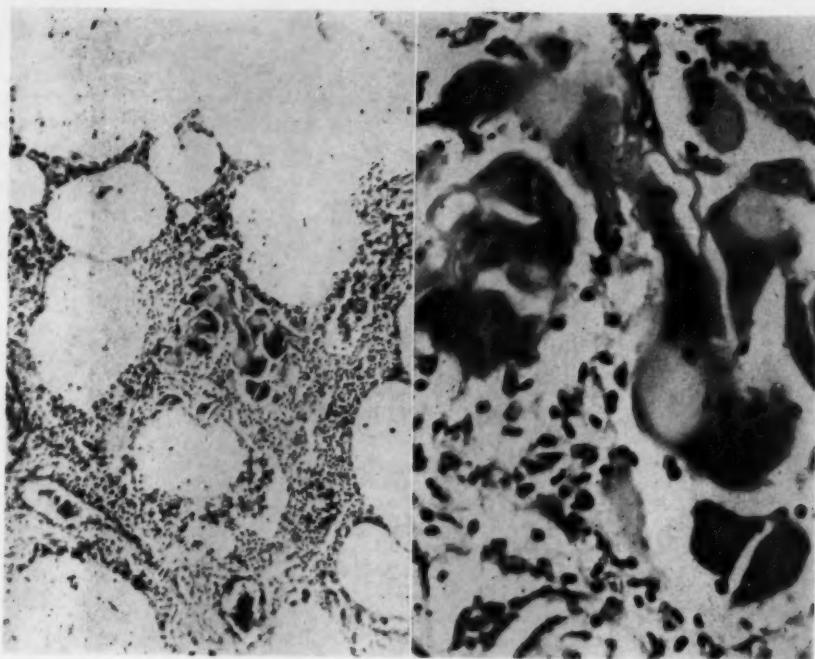


Fig. 3

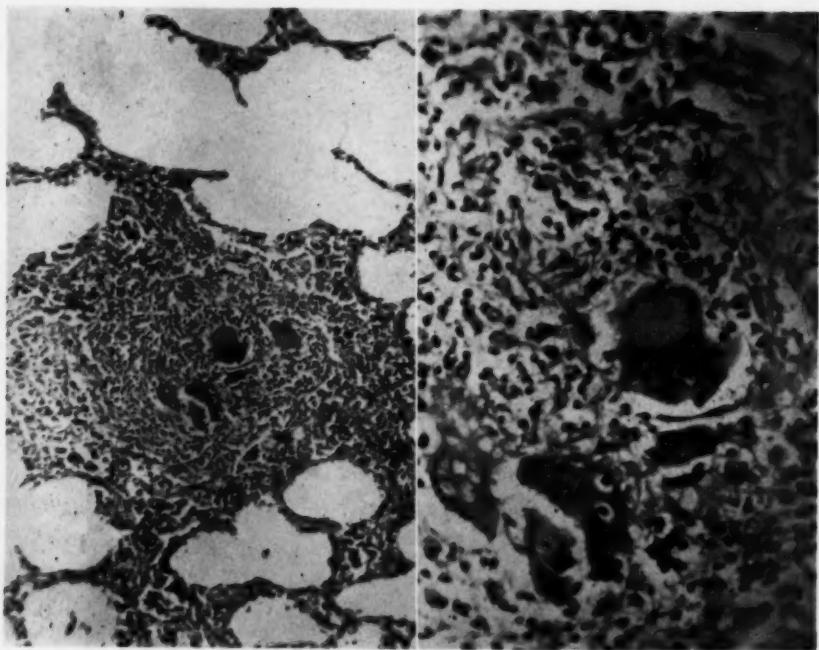


Fig. 6

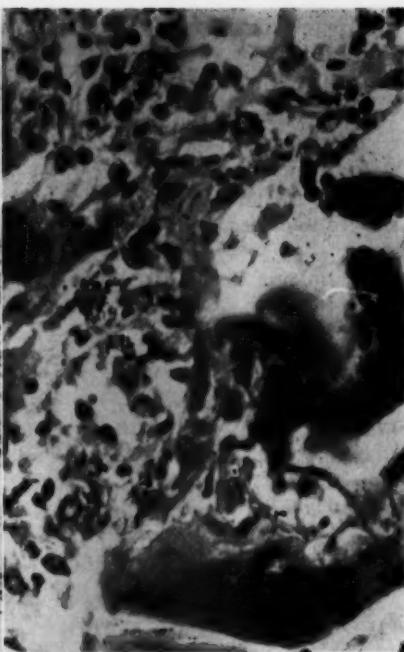
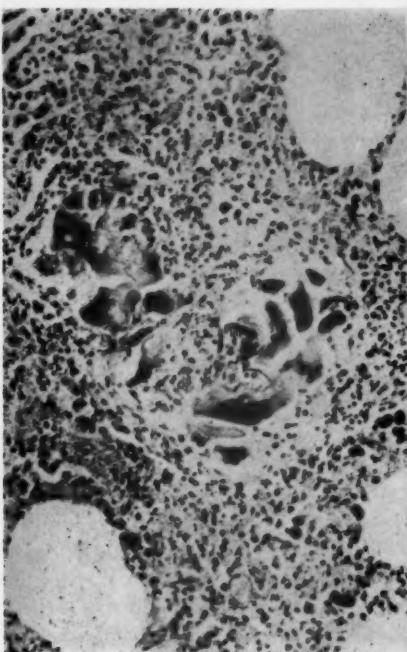
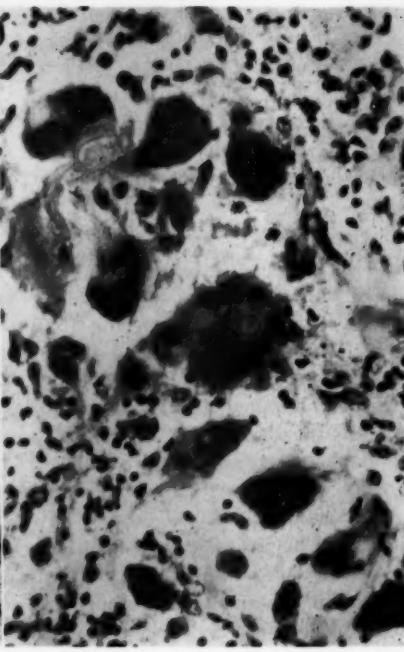


Fig. 5



**Brain.**—There was a moderate degree of congestion with perivascular cuffs of lymphocytes; the meninges were normal.

**Other Tissue.**—Histologic examination of sections taken from heart, intestines, muscles, lymph nodes, and skin revealed no abnormality. Sections of the kidney and spleen showed some degree of congestion.

#### Parasitologic Examinations

Fresh tissue obtained from the patient at necropsy was digested in pepsin as follows: 1.5% pepsin in saline, adjusted to pH 1.5 with hydrochloric acid, was added with portions of tissue to a Waring Blender cup in the proportion of 20 cc. of pepsin solution to 1 gm. of tissue. After thorough comminution, the mixture, which was transferred to conical sedimentation flasks, was incubated at 37°C for eight hours and the digest examined for larvae; 50 gm. of lung, 90 gm. of liver, 10 gm. of heart, 10 gm. of diaphragm, and 20 gm. of skeletal muscle were digested with pepsin, but no larvae were found; 100 gm. of lung tissue subdivided into three lots was placed in Baermann's apparatus overnight; the sediment examined next morning did not show any larvae. Examination of press preparations of lymph nodes also failed to reveal any larvae. Histologic examination of serial sections from several blocks of lung and liver tissue did not reveal any parasites.

#### Biopsy Studies

**Liver.**—Serial sections were made from tissue obtained by needle biopsy of the liver in 12 patients diagnosed as suffering from eosinophilic lung and from portions of liver obtained at laparotomy from 2 others. All these patients presented with symptoms of cough and breathlessness and had a hypereosinophilia varying from 6,000 to 27,000 cells per cubic millimeter. Night blood examinations did not reveal any microfilariae. The filarial complement-fixation test which was done in three of them was positive in titers varying from 1:10 to 1:320. All the patients responded

satisfactorily to treatment with diethylcarbamazine; the positive FCFT titers subsequently became negative.

A study of the sections from the liver biopsy specimens showed that the architecture was normal, but in the periportal areas there was a moderate infiltration with lymphocytes and cells morphologically resembling histiocytes and fibroblasts (Figs. 7A and B). Scattered among these cells and in the sinusoids were a varying number of eosinophils. No granulomas were seen in any of the sections, and a careful search did not reveal the presence of any parasites.

In view of the possibility of a filarial etiology, it was decided to examine liver tissue from cases of filariasis and compare the findings with those given above. Needle biopsies of the liver were performed on eight cases of Bancroftian filariasis, and the material obtained was serially sectioned. No parasites or focal granulomas were seen on histologic examination, and the normal architecture of the liver was preserved, but in the periportal areas similar infiltrations with lymphocytes and cells morphologically resembling histiocytes and fibroblasts were found (Figs. 8A and B).

**Lymph Node.**—Six patients suffering from eosinophilic lung had small palpable lymph nodes in the inguinal or axillary areas. They presented with cough and breathlessness and had a hypereosinophilia varying from 6,000 to 30,000 cells per cubic millimeter. Night blood examinations for microfilariae were negative, but the FCFT done in five of them was positive in titers varying from 1:10 to 1:80. All of them responded to treatment with diethylcarbamazine, and the positive FCFT titers subsequently became negative. The lymph nodes were removed and sectioned serially. Histologic examination showed that the architecture of the nodes was well preserved, but a moderate nonspecific follicular hyperplasia with a varying degree of infiltration by eosinophils was seen in some of the sections. No parasites were found, and the histologic picture was not suggestive of filariasis.

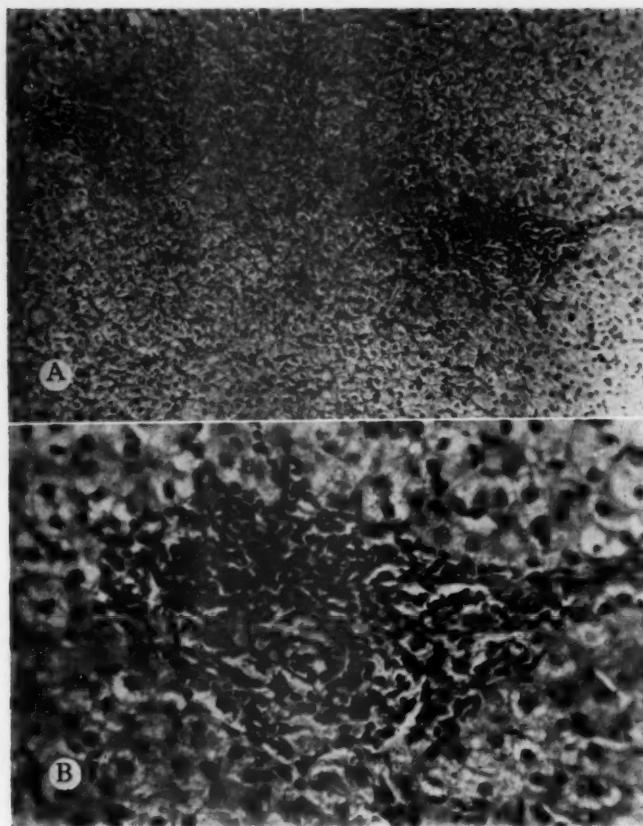


Fig. 7.—*A*, liver in a case of eosinophilic lung. Infiltration with lymphocytes and cells morphologically resembling histiocytes and fibroblasts in the periportal areas. *B*, higher magnification of *A*. Hematoxylin and eosin stain; *A*,  $\times 100$ ; *B*,  $\times 330$ .

#### Comment

Knowledge of the histopathologic features of eosinophilic lung has so far been limited to two necropsy reports.<sup>19,20</sup> In these cases death occurred as a result of encephalopathy following treatment with organic arsenicals, and so the original pathologic picture was probably altered by the drug. Excluding the congestive and hemorrhagic changes in the lungs and brain as probably being related to drug intoxication, the pertinent features reported by Viswanathan were found in the lungs and consisted of scattered areas of interstitial fibroblastic proliferation and eosinophilic infiltration with macrophages in some of the adjacent alveoli. A striking feature was the presence of granulomatous lesions consisting of large multinucleated giant cells in the center sur-

rounded by monocytes; the nuclei in each of the giant cells varied from 15 to 25 in number and were all gathered together in the center. Viswanathan concluded that the histologic appearance was suggestive of an infective process rather than an anaphylactic reaction. Granulomas were not seen in the second necropsy case,<sup>20</sup> the histologic changes in the lungs being essentially bronchial and peribronchial eosinophilic infiltrations.

In the necropsy case reported in this paper, the most significant feature was the focal granulomatous reaction in the lungs similar to that described by Viswanathan.<sup>19</sup> The lesions were not suggestive of tuberculosis, and the giant cells were considered to be of the foreign-body type. It is interesting to speculate on the nature of

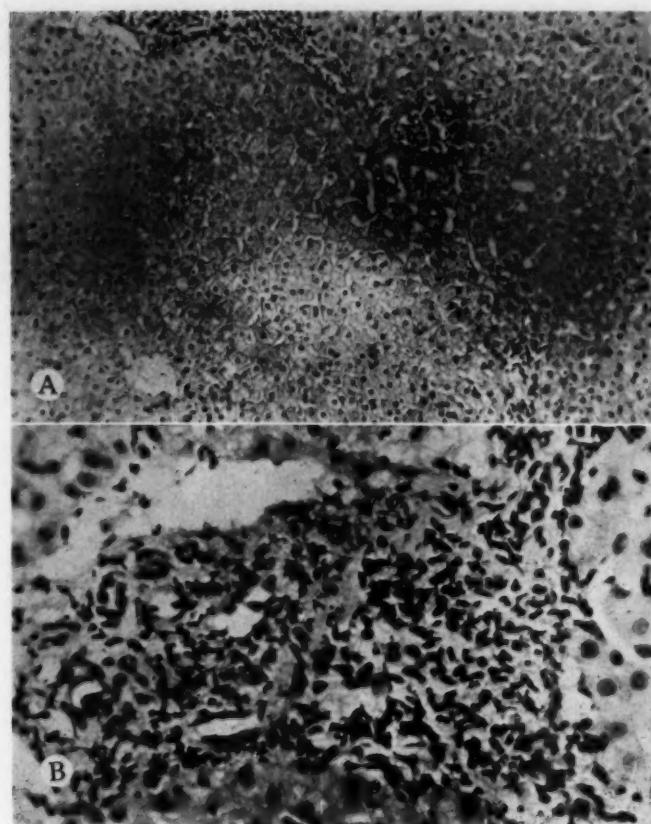


Fig. 8.—*A*, liver in a case of Bancroftian filariasis. Infiltration with lymphocytes and cells morphologically resembling histiocytes and fibroblasts in the periportal areas. *B*, higher magnification of *A*. Hematoxylin and eosin stain.

the acidophilic necrotic material in the center of the lesions and consider it to be disintegrating larvae, but in no section was it distinctive enough to be identified as parasites. The patient having been treated, it is unlikely to find larvae in tissue, although evidence of their invasion was present in the form of foreign-body granulomas. As these lesions were not situated in relation to bronchioles, it is likely that the infecting agent entered the lung tissue via the blood stream.

The liver biopsy studies in the series of eosinophilic lung and filariasis cases revealed an essentially similar feature, viz., a periportal lymphocytic infiltration nonspecific in nature. Sections of liver obtained from the necropsy case also showed the same change. Other workers<sup>11,16</sup> have observed similar

infiltrations in the portal tracts and sinusoids of liver tissue obtained by needle biopsy in cases of eosinophilic lung.

Foreign-body granulomas similar to the type described above have been reported in cases of toxocariasis,<sup>21</sup> but they have usually been seen in the liver although they may be found in any part of the body, including the lungs.<sup>22,23</sup> In a case of pulmonary ascariasis<sup>24</sup> in which the larvae were found in the bronchioles and identified as *Ascaris*, probably *A. lumbricoides*, the histologic features consisted of granulomatous lesions in the liver and changes in the lungs compatible with chronic bronchitis and bronchial asthma; no granulomas were found in sections of the lung. However, extensive serial sections of liver tissue from the necropsy case reported in this paper and

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that obtained by biopsy did not reveal any granulomas. Hence it is probable that the main histologic finding in cases of eosinophilic lung is confined to the lung and consists of foreign-body granulomas formed around the invading agents.

In view of the reports of the finding of microfilariae and tissue changes suggestive of filariasis in the lymph nodes of a few patients with hypereosinophilia, lymphadenopathy, and pulmonary symptoms,<sup>25-30</sup> histologic examination of lymph nodes was carried out. No microfilariae or adult worms were found in extensive serial sections, nor was the histologic picture one of filariasis. Eosinophilic lung is not associated with the gross lymphadenopathy seen in filariasis nor with any of the other classical clinical features of the latter disease. Furthermore, blood examination for microfilariae (with use of Knot's concentration technique) in more than 300 cases of eosinophilic lung have consistently been negative.<sup>31</sup> Nevertheless, the positive serologic reactions obtained with the filarial complement-fixation test<sup>17,18</sup> suggest the etiologic possibility of a filarial type of infection which has not been described previously due to the absence of microfilaremia at any stage.

### **Summary**

A complete pathologic study of an adult Ceylonese man who died of encephalopathy following treatment of eosinophilic lung with neoarsphenamine is reported. The significant histologic finding was the presence of foreign-body granulomas in the lungs. In a series of liver biopsies granulomatous lesions were not found, but there was periportal infiltration with leukocytes which was considered to be nonspecific. Similar changes were found in liver biopsy specimens from cases of filariasis. Lymph node biopsy specimens did not reveal any significant abnormality.

Dr. H. Maycock performed the necropsy; Dr. Wong Poi Kwong did the needle liver biopsies; Dr. K. Vellasamy performed the open operation liver and the lymph node biopsies; Dr. K. Shanmugaratnam, Senior Pathologist, Singapore, helped

with the histopathologic study; Prof. P. C. Beaver and Mr. J. F. Schacher, of Tulane University, did the parasitologic examinations reported in this paper; Mr. V. Nalpon took the photomicrographs. This study was supported in part by Armed Forces Epidemiological Board, Commission on Parasitic Diseases.

Department of Medicine, University of Malaya.

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## Extracellular Distribution of Ferrocyanide in Muscle

WILLIAM H. CHASE, M.D., Chicago

The ground substance of connective tissue has been studied in tissue sections with methods designed to preserve water-soluble substances. In tissue prepared by freezing and drying ground substance is stainable by the periodic acid-Schiff (PAS) technique. In the light microscope it appears to be uniformly dispersed between the cells and fibers of the connective tissue save for condensation as basement membrane.<sup>1,2</sup> Gersh and Catchpole<sup>1</sup> described changes of the ground substance in different conditions, changes which have been interpreted as reflecting corresponding changes in the aggregation of components of ground substance.

Electrochemical studies by Joseph, Engel, and Catchpole<sup>3-5,15</sup> provide data on the concentration of immobile, negatively charged colloids in the connective tissue. From thermodynamic considerations the colloids were said to be organized as a two-phase system: a colloid-rich (water-poor) and a colloid-poor (water-rich) phase. This situation provides the minimum but sufficient requirement for any connective tissue to be in equilibrium with the blood. The two phases themselves are also in thermodynamic equilibrium. The actual dimensions and morphological disposition of the two phases could not be stated from physicochemical data.

It has seldom been possible to examine the ground substance with the electron microscope because of its low density and also because it is probably largely dissolved in the commonly used aqueous osmium tetroxide fixatives. Using the freeze-dry technique, Bondareff<sup>6</sup> showed that the

ground substance of the rat-tail tendon appears in the electron microscope as a vacuolar system. The contents of the vacuoles were found to have a low density and were surrounded by a continuous phase of greater density. The work of Bondareff thus offers a morphological basis for the two-phase system postulated on theoretical grounds.

The purpose of this paper is to describe the distribution of injected ferrocyanide in the extracellular ground substance of the muscle in the mouse diaphragm. The choice of mouse diaphragm for this study was dictated by the convenience with which good fixation could be obtained without cutting muscle or ground substance. Incidentally, the preparation is also particularly appropriate because of the large literature which exists on metabolic studies with the diaphragm.

The findings indicate that the water-rich phase of the ground substance exists at least partly as submicroscopic vacuoles surrounded by the colloid-rich (water-poor) phase. Ferrocyanide ion diffuses into and is possibly concentrated in the vacuoles. If precautions are taken to prevent diffusion during the test for ferrocyanide, its distribution in the vacuoles may be seen with the light and electron microscope in sections of frozen-dried tissue by means of the Prussian blue reaction. The surrounding fraction of the ground substance is not stained.

There is a large literature on the effects of insulin on the diaphragm. Nearly all of it is concerned with possible effects of the hormone on the muscle fibers. These metabolic aspects have been reviewed by Stadie<sup>7</sup> and Krahl.<sup>8</sup> It was decided to test by means of the ferrocyanide method whether insulin may also induce changes in

Submitted for publication Aug. 28, 1958.

Department of Anatomy, The University of Chicago. Fellow of the Helen Hay Whitney Foundation.

the ground substance of the diaphragm which encloses the muscle fibers and constitutes their immediate environment.

### Experimental

Fifty adult white mice were used in the study. A 20% solution of ferrocyanide was prepared just before injection, consisting of 19.0 gm. of the sodium salt and 1.1 gm. of the potassium salt per 100 ml. of solution. Most of the animals received 0.3 ml. of the ferrocyanide solution, given slowly via the tail vein over a period of about three minutes.

In one series of mice the dose ranged from 0.1 ml. to 1.0 ml., which is about as much as they would tolerate. Within one or two minutes after injection the animals were bled through the abdomen. The abdominal organs were quickly removed; the rib cage was cut along the diaphragmatic insertion and the pericardial attachments severed. The xiphoid process and adjacent tissue were removed, exposing the anterior diaphragmatic insertion. While the diaphragm was held with forceps away from adjacent organs, and in as normal state of stretch as possible, the diaphragm was frozen as liquid propane, cooled to about -175°C in liquid nitrogen, was poured on it. Rewarming was prevented with immediate pouring of liquid nitrogen over the area and then the entire animal being plunged quickly into liquid nitrogen. The anterior part of the diaphragm was then chipped off under liquid nitrogen with chilled bone forceps.

Fragments of the muscular diaphragm 3-5 mm. square were dried for 24 hours in a vacuum at a temperature of about -35°C with use of the method described by Finck.<sup>9</sup> No post fixation was used. It is noteworthy that in the subsequent handling of the tissue great care must be taken to exclude moisture. The tissues were infiltrated with paraffin (58°C) in vacuo and imbedded. Sections were cut at 10 $\mu$  and applied to lightly albuminized slides with finger pressure. The sections were then warmed gently over a microburner sufficient just to melt the paraffin and then pressed down again with the finger.

The Prussian blue reaction was carried out in a manner designed to minimize diffusion until the insoluble ferric-ferrocyanide had been formed. Use of fresh xylene kept from contact with moist air was found to be especially important. The xylene was saturated with anhydrous ferric chloride in a clean dry Coplin jar, which was kept covered. When bubbling almost ceased (5-10 minutes), the slides were immersed in the solution for three minutes. The slides were then transferred successively to absolute alcohol, 95% alcohol, and distilled water, each of these solutions being nearly

saturated with hydrated ferric chloride. The slides were kept for about three minutes in each solution, with frequent agitation. They were then washed in three changes of absolute alcohol, taken to xylene, and mounted in Permount. The method is essentially the same as that described by Gersh and Stieglitz.<sup>10</sup>

Some tissues were prepared for electron microscopy by scraping off the stained sections from the slide with a clean razor blade in 95% alcohol. The fragile sections were collected with a pipette and transferred to absolute alcohol. After three changes they were imbedded in methacrylate. Several dozen sections were imbedded at random in one block.

Ten mice were given injections subcutaneously with a solution containing 0.1 $\mu$  crystalline insulin about one hour before ferrocyanide was injected and the diaphragm frozen.

### Results

In sections of mouse diaphragm studied with the light microscope the ferrocyanide is seen as small blue droplets which are dispersed singly or in small clusters in the connective tissue between the muscle bundles. The size of these droplets is about 0.25 $\mu$ . Larger aggregations are often present, but on close inspection these are seen to have a lobulated appearance and are evidently composed of clusters of smaller droplets which cannot be clearly resolved. The intensity of the blue precipitate is not uniform among the droplets but varies from a dark blue to a light color which is only faintly visible. Larger doses of ferrocyanide increase the density of the stain but do not appear to alter the droplets in any other way. The intervening ground substance does not show any blue, even with the highest doses of ferrocyanide used. A dose of 0.1 ml. of ferrocyanide renders the vacuoles just barely visible; below this dose ferrocyanide cannot be detected at all in the ground substance.

In the electron microscope the ferrocyanide droplets, which are very dense, appear as circular or spheroidal structures with relatively sharp margins. These vacuoles measure 600 to 1,200 Å. in diameter. Several may be aggregated to form grape-like clusters, but the individual vacuoles are clearly outlined in normal con-

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nective tissue. The droplets appear homogeneous with the electron microscope when the pictures are taken at low beam intensity, but a high beam intensity seems to cause a separation of the precipitate into small granules of about 80 Å.

In the animals treated with insulin the distribution of the ferrocyanide is altered. The vacuoles are larger and many appear to have coalesced, forming lakes or pools of blue. The intervening ground substance is not stained.

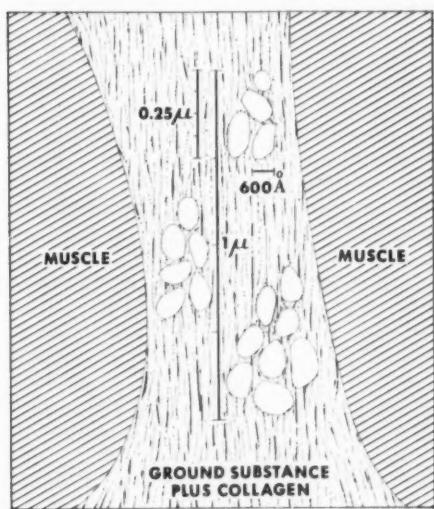
### Comment

Recent studies<sup>11</sup> showed that ferrocyanide is almost entirely free and unbound in plasma in the doses employed in this study. Calculations have shown that the extracellular volume in the tissue occupied by the ferrocyanide is not very different from that occupied by inulin or creatinine.<sup>11-13</sup> An assumption of these calculations is that ferrocyanide should be uniformly distributed in the extravascular tissue spaces. The

observations reported in this study show that this assumption is unwarranted. Ferrocyanide is observed to be unevenly distributed in the ground substance, occurring as droplets or clusters of droplets where the concentration is at least 10 times more than that in the intervening ground substance (Fig. 1). This finding involves a modification of the concept of "tissue fluid" and "tissue spaces" somewhat along the lines suggested by Gersh and Catchpole,<sup>1</sup> Gersh,<sup>2</sup> and Catchpole, Joseph, and Engel.<sup>4</sup> The former considered tissue fluid to be coextensive with the ground substance. The latter have postulated a two-phase organization of the ground substance into colloid-rich and water-rich components in which the "tissue fluid" becomes an integral part of the structure of ground substance. Such structure was visualized by Bondareff in the rat-tail tendon.

The explanation of the peculiar distribution of ferrocyanide in the connective tissue of the mouse diaphragm is not altogether simple, especially in view of the more uniform distribution of chloride in the ground substance (Gersh).<sup>14</sup> Of the several possible explanations, the following one seems to fit most of the findings and derives support from data in the literature: Ferrocyanide ions have a high negative charge and are strongly repelled from regions which have a high negative charge but are less strongly repelled from regions which are less highly negatively charged. This would explain the accumulations in higher concentrations in the water-rich phase of the ground substance.

The absence of staining of the ground substance between the vacuoles even with the largest doses of ferrocyanide which the animal would tolerate must indicate a marked partition of the ferrocyanide ion in favor of the vacuoles, since none is seen in the dense phase of the ground substance after a dose of 1.0 ml., when the concentration of ferrocyanide in the vacuoles is highest. A dose of 0.1 ml., on the other hand, renders the vacuoles just barely visible.



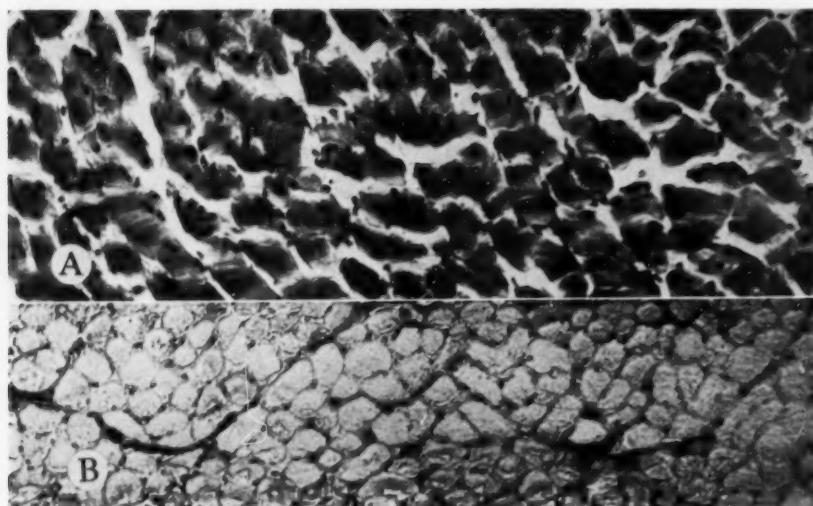
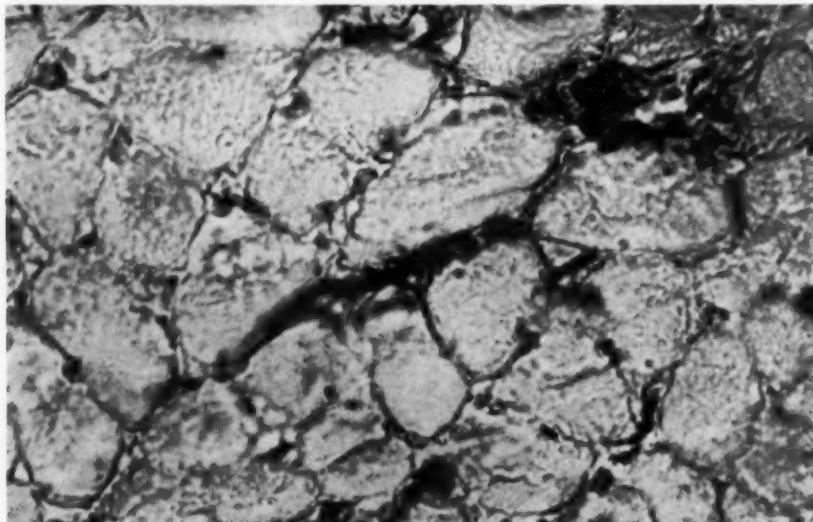


Fig. 2.—Photomicrographs of mouse diaphragm prepared by freezing and drying after the animal was given injections of ferrocyanide solution. *A*, stained with hematoxylin and eosin to show densely stained muscle fibers surrounded by pale intercellular connective tissue. *B*, the result of the Prussian blue reaction only. The muscle fibers are unstained, while the dark Prussian blue identifies ferrocyanide in the intercellular ground substance. Both photomicrographs  $\times 300$ .

Observations indicate a changing pattern of organization of the ground substance of the connective tissue of the diaphragm

under the action of insulin. The droplets containing ferrocyanide cannot be regarded as permanent structures. What is indicated

Fig. 3.—Photomicrograph of mouse diaphragm prepared by freezing and drying after the animal was given injections of ferrocyanide solution. The darkly stained areas identify ferrocyanide in the extracellular ground substance. The ions occur as droplets or larger aggregations. The muscle fibers are not stained;  $\times 600$ .



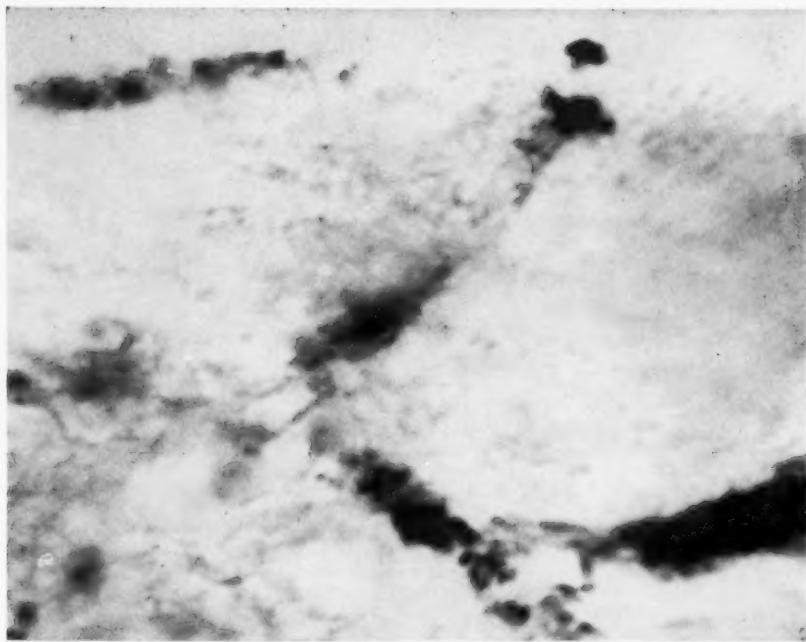
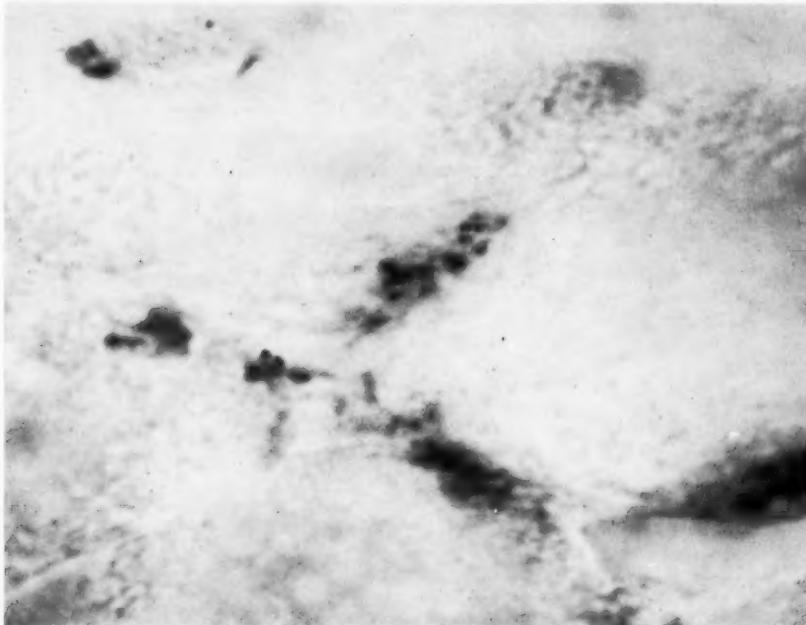


Fig. 4.—Same section as Figure 3. The ferric ferrocyanide appears as more or less discrete droplets in the connective tissue between the muscle fibers. In the connective tissue between the droplets no ferrocyanide is visible;  $\times 1,200$ .

Fig. 5.—Same field as Figure 4, slightly different focus. Regions which appeared to be diffusely stained by Prussian blue in Figure 4 show rather discrete droplet localization of the ferrocyanide. Other regions, however, have become apparently diffuse;  $\times 1,200$ .



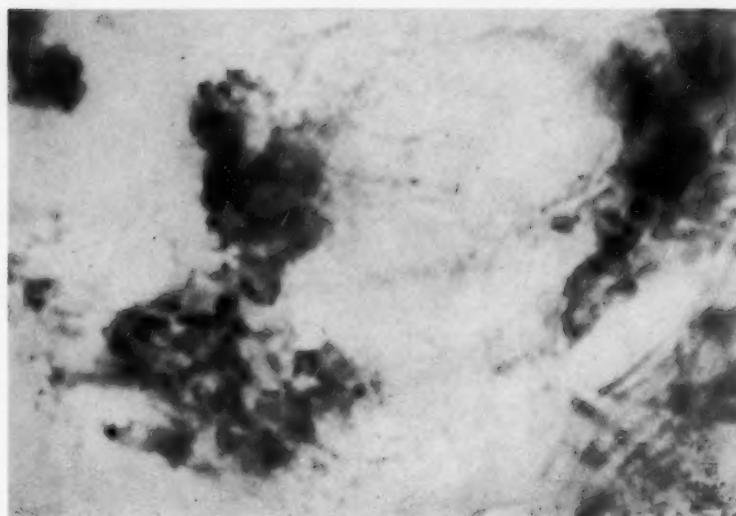
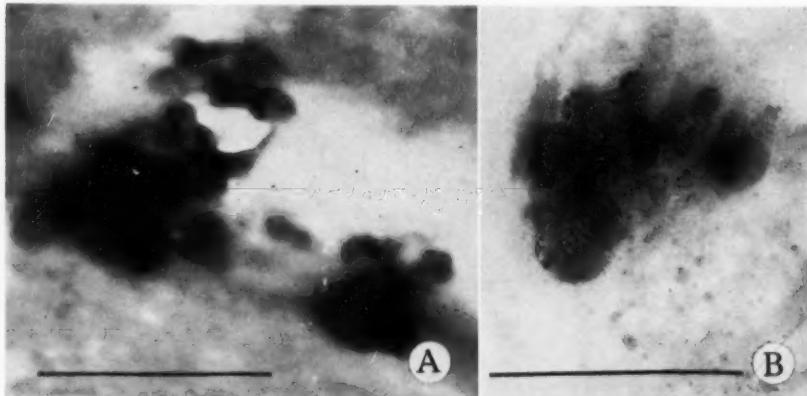


Fig. 6.—Section of diaphragm prepared as in Figure 3. This animal was given an injection of insulin about one hour before the diaphragm was frozen. The Prussian blue reaction shows a more diffuse localization of ferrocyanide in the extracellular space. Droplets are seldom discrete and often appear to be fused together, forming pools of blue. This appearance suggests an altered state of equilibrium in the ground substance colloids;  $\times 1,200$ .

is, rather, a constantly changing state of organization of the ground substance. This interpretation is fortified by the findings of Dennis.<sup>16</sup> Most generally, there is a decrease in the amount of colloid-rich phase, leading to a greater fluidity. This

state is presumably reversible (Joseph, Engel, and Catchpole).<sup>15</sup> With a shift to the water-rich state, there is a corresponding lowering of the colloidal charge density of the matrix as a whole and a lowering of its electrolyte content. However, by virtue

Fig. 7.—Electromicrographs of diaphragm, prepared as described in Figure 3, followed by methacrylate embedding and thin sectioning; *A*, taken at low beam intensity. The Prussian blue appears as droplets having high density and measuring 600-1,200 Å. in diameter. Bar at bottom indicates 1  $\mu$ . *B*, taken at high beam intensity, showing separation of the ferric ferrocyanide precipitate into granules of about 80 Å., apparently caused by the electron beam. The distribution of ferrocyanide in droplets is still clearly evident, however. Bar at bottom indicates 1  $\mu$ .



## FERROCYANIDE IN MUSCLE

of the persisting two-phase relationship, the tissue remains in equilibrium with the blood.

In this paper, a change is demonstrated in the relative proportions of the protein-rich and water-rich phases of ground substances as a consequence of giving injections of insulin to the animal. Such a change has not been sought for, or assumed, in any of the experiments designed to study the effects of insulin on muscle. The effects of other hormones and other treatments on the structure of ground substance are presented in an accompanying paper by Dennis.<sup>16</sup>

### Summary

The distribution of ferrocyanide in the connective tissue of the mouse diaphragm has been studied by means of freezing and drying and the Prussian blue reaction. The findings indicate that ferrocyanide is not uniformly distributed in the ground substance but occurs as small droplets or aggregations of droplets. Between the droplets, ferrocyanide could not be detected in the ground substance. In the electron microscope the droplets were seen to be resolved into still smaller vacuoles measuring 600-1,200 Å. in diameter.

These findings are interpreted in terms of a two-phase organization of ground substance, which has been postulated by the electrochemical studies of Joseph, Engel, and Catchpole. The distribution of ferrocyanide is believed to identify the water-rich phase of the ground substance, which is in the form of vacuoles surrounded by the colloid-rich phase.

A change is demonstrated in the relative proportions of the two phases of the ground substance as a consequence of giving the animal injections of insulin. This finding, together with those of Dennis in an accompanying paper,<sup>16</sup> is indicative of a constantly changing state of organization of the ground substance.

This work was performed under the supervision of Dr. Isidore Gersh. Dr. Hubert R. Catchpole

assisted in the preparation of the manuscript.

The expenses for this study were defrayed by grants from the Clara A. and Wallace C. Abbott Memorial Research Fund, the Helen Hay Whitney Memorial Research Fund, and the Commonwealth Fund.

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# Effects of Various Factors on the Distribution of Ferrocyanide in Ground Substance

JOEL B. DENNIS, M.D., Chicago

In another paper,<sup>1</sup> Chase applied a method which makes it possible to see in sections the manner in which ferrocyanide is distributed in the ground substance of the connective tissue of diaphragmatic muscle. In normal untreated animals, visible ferrocyanide is distributed as minute vacuoles which have a submicroscopic structure. These vacuoles appear to represent at least in part the water-rich phase of ground substance. Between the vacuoles where ferrocyanide is not visible, the ion is believed to be present but in a concentration too low to be detected by the method employed. This region is interpreted as representing largely the water-poor phase of the ground substance. Both phases are assumed to be in thermodynamic equilibrium. Chase showed that the relative amounts of the two phases were altered by treatment of the animals with insulin. For this report, additional factors which influence the relation of the two phases have been studied and the results are described. In general, it may be said that a wide variety of treatments influence the distribution of ferrocyanide in the ground substance and hence of the water-rich phase in particular. These include hydration, dehydration, denervation, inflammation, aminoacetonitrile, various compounds having hormonal effects, such as *l*-triiodothyronine, estrogen, deoxycorticosterone (DOCA) acetate, cortisone, parathyroid hormone (Parathormone), and ascorbic acid (vitamin C). The effects of rickets induced by feeding a high-calcium, low-phosphorous, vitamin D-depleted diet are also marked.

Submitted for publication Aug. 28, 1958.

Departments of Surgery (Orthopedics) and Anatomy, The University of Chicago.

Prominent changes which take place in the water-rich phase of the ground substance during growth and aging of the animals are also described.

## Materials and Methods

Mice were treated in a variety of ways before injection of ferrocyanide intravenously. The distribution of ferrocyanide in the diaphragm of these animals was compared with that of control animals given similar injections but no prior treatment. Young adult male albino mice (25-30 gm.) were used in all experiments except as indicated.

The mice were given injections over a three-minute interval with 0.02 cc. per gram of a 20% aqueous solution of ferrocyanide. The animals were bled; the diaphragm was exposed and frozen with propane chilled to about -175°C. Small pieces were dried in vacuo at about -35°C, embedded in paraffin, and sectioned at 10 $\mu$  transversely to the axis of the muscle fibers. The sections were then tested for ferrocyanide and mounted in Permount. The ferrocyanide appeared as Prussian blue which was readily observed with the microscope. The details of the whole procedure were described by Chase. In this section is described only the treatment of the mice before the injection of ferrocyanide.

One series of rats was studied for the effect of a rachitogenic diet on the ground substance of connective tissue. Details are reported under Section P, below.

In addition, some preliminary studies were made on diaphragms removed from the mice and incubated in a buffered Ringer solution containing ferrocyanide. Details of the *in vivo* and *in vitro* experimental conditions are given separately.

## I. Treatment of Mice Prior to Injection of Ferrocyanide—*in Vivo* Experiments

A. Normal, Untreated, Aging Series.—1. White Mice: Untreated littermates were used. They were killed in groups of four at the ages of 4, 8, 12, 24, 30, 37, and 45 days. These were compared with four white mice (NIH strain) 540 days old. The latter had roughened, thin, coarse fur, with missing or broken vibrissae, and were inactive. Ferro-

cyanide was injected into the younger mice directly into the heart; it was injected intravenously in the others.

**2. LAF<sub>1</sub> Strain:** This strain is a hybrid (C 57 Leaden/HE ♀ × Strain A/He ♂) with longevity greater than that of the NIH strain. Two age groups given injections with ferrocyanide were compared with each other and with the white mice: 5 at 70 days and 3 at 1,095 days. The older group was active and had smooth, glistening fur with vibrissae characteristic of young animals.

**B. Hydration.**—Mice were given injections at 30-minute intervals intraperitoneally with 5-10 cc. of a 0.9% sodium chloride solution. Each mouse received 35 ml. of this solution in three hours. Ferrocyanide was then injected.

**C. Dehydration.**—Three mice were fed for seven days on oven-dried Dixie Dog Food, with no added water. They were then given an injection of ferrocyanide.

**D. Denervation.**—The phrenic nerves in a large number of mice were cut bilaterally at the thoracic inlet. Two survived five days when they were given ferrocyanide.

**E. Inflammation.**—Seven mice were anesthetized and the diaphragm exposed through a right subcostal incision. The right half of the abdominal surface of the diaphragm was scarified with a sharpened toothpick and the abdominal wall closed. Two days later the animals were given injections of ferrocyanide. The uninjured side of the diaphragm served as the control for the injured side.

**F. Aminoacetonitrile.**—Eight mice were fed for four weeks powdered Dixie Dog Food which contained 0.25% of aminoacetonitrile hydrogen sulfate (Abbott Laboratories) and then given injections with ferrocyanide.

**G. Liothyronine (*I*-Triiodothyronine).**—Each of five mice was given a subcutaneous injection daily for five days of a suspension of 5 µg. of sodium liothyronine (Cytomel; Smith, Kline & French Laboratories). Three control mice were given injections in the same way of salt solution. All were given ferrocyanide at the fifth day.

**H. Aminoacetonitrile and Liothyronine.**—Eight mice were fed powdered Dixie Dog Food which contained 0.25% aminoacetonitrile and 3.5×10<sup>-6</sup>% of the iodinated compound. Three mice survived four weeks and were given ferrocyanide injections.

**I. Estrogen.**—Ten mice received daily for five days subcutaneous injections of 1,000 R.U. of estradiol benzoate in sesame oil (Progynon-B, Schering Corporation). Control mice were given similar injections, but only with sesame oil. Ferrocyanide injection was given to both groups at the end of five days.

**J. Deoxycorticosterone Acetate.**—Five mice were given injections subcutaneously daily for eight days with 1 mg. of deoxycorticosterone acetate

(The Upjohn Company). Two control mice were given similar injections but with saline. Ferrocyanide was injected into both groups at the eighth day.

**K. Cortisone.**—Ten mice received daily subcutaneous injections of 3 mg. of cortisone acetate (Cortogen acetate; Schering Corporation) for five days. Four control animals received saline by the same route. After this treatment ferrocyanide was injected.

**L. Ascorbic Acid.**—Each group of five mice was given subcutaneous injections daily for 14 days with 0.4, 2.0, and 10.0 mg. of ascorbic acid in salt solution. Three control mice were given injections of saline for the same time. All mice were then given injections of ferrocyanide.

**M. Cortisone and Ascorbic Acid.**—Five mice were given injections subcutaneously daily for five days with 10 mg. of ascorbic acid and 5 mg. of cortisone. They were then given injections of ferrocyanide.

**N. Vitamin A.**—Each group of five mice was given subcutaneous injections daily for 14 days with 1, 5, and 25 I. U. of water-miscible vitamin A, as Aquasol A (U. S. Vitamin Corporation). Three control mice were given injections in the same manner with saline. All were then given injections of ferrocyanide.

**O. Parathyroid Hormone.**—Eight mice were given injections twice at 12-hour intervals intraperitoneally with 250 U. S. P. units of parathyroid hormone (Eli Lilly and Company). They were then given injections of ferrocyanide. Four mice were given saline in the same way and served as controls.

**P. Rachitogenic Diet High in Calcium, Low in Phosphorus, and Depleted of Vitamin D.**—Sixteen male Sprague-Dawley weanling rats were fed Rachitogenic Diet No. 2 (Nutritional Biochemical Corp.). The rats were kept in separate cages in a darkened room and drank distilled water. Four controls were fed Dixie Dog Pellets. After 25 days, when marked rachitic changes in the tibia of the experimental rats were evident on x-ray films, the rats were given injections of ferrocyanide. Each animal received intraperitoneally 0.02 ml. per gram of body weight of the 20% ferrocyanide solution. This was repeated five minutes later. The controls were given the same dose of ferrocyanide. All rats were killed five minutes after the second injection.

## II. In Vitro Preparations of Isolated Rat and Mouse Diaphragm Incubated in Ferrocyanide Solution

Two kinds of diaphragm preparations were studied: intact diaphragm and cut diaphragm.

**A. Intact Diaphragm.**—After the animals were bled the diaphragm was removed with an adhesive

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margin of rib cage to avoid the cutting of muscle fibers.

*B. Cut Diaphragm.*—The diaphragm was removed, and muscle fibers were cut at their origin, with the attachment at the central tendon intact.

*C. Preparation.*—The diaphragm was then incubated in a closed flask containing 20 ml. of preoxygenated medium at 38°C, with gentle agitation. The medium consists of the following compounds (in grams) dissolved in 460 ml. of distilled water and then brought to pH 7.4 with 0.1 M phosphate buffer:

NaCl 1.8	MgSO <sub>4</sub> · 7H <sub>2</sub> O 0.152
KCl 0.092	Na <sub>2</sub> Fe(CN) <sub>6</sub> · 1OH <sub>2</sub> O 6.92
CaCl <sub>2</sub> 0.146	K <sub>4</sub> Fe(CN) <sub>6</sub> · 3H <sub>2</sub> O 0.42

*D. Procedures.*—The experiments with these preparations include the following procedures.

1. *Mouse Intact Diaphragm.*: The diaphragms of three mice were incubated *in vitro* for each time period (in minutes): 10, 20, 30, 45, 60, 120, and 420. At the end of each period, the diaphragm was stretched gently between fine wire retractors and frozen by pouring over it liquid propane, chilled to about -175°C. Small pieces were dried at low temperature (-35°C), embedded in paraffin, sectioned, and tested for ferrocyanide in the usual manner.

2. *Rat Intact Diaphragm, Prepared as Above and Incubated for Sixty Minutes*: Three rat diaphragms were used.

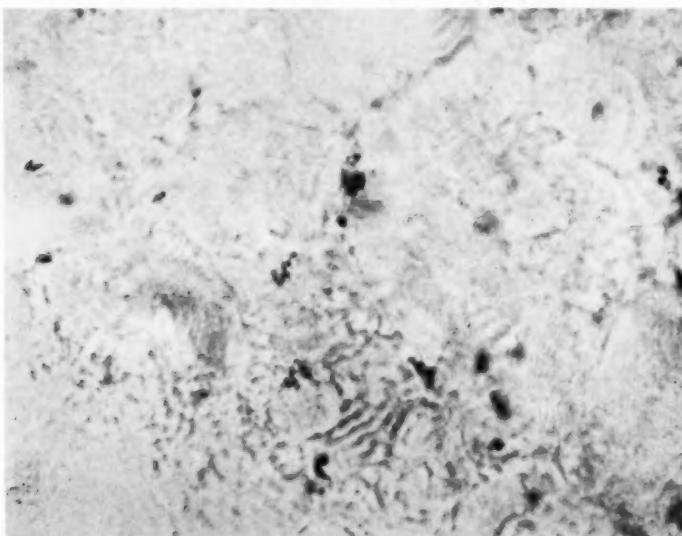
3. *Rat Cut Diaphragms, Incubated for 30, 60, 90, and 120 Minutes* (three at each time interval): These were then frozen and dried in the usual way and cut longitudinally in order to estimate the rate of diffusion of ferrocyanide within the muscle fibers inward from the cut surface of the diaphragm.

## Results

Ferrocyanide is visible as Prussian blue in the ground substance spaces in the form of minute droplets which occur singly or in clusters. These are separated from each other by regions of extracellular tissue where no ferrocyanide is visible. Muscle fibers are also free of visible ferrocyanide. The range of variation in the normal young adult is given in Figures 1 and 2. The droplets containing ferrocyanide are in the ground substance of the connective tissue.

*In Vivo.*—In the series of *in vivo* preparations considered as a whole, the number, size, and relation of the ferrocyanide droplets vary markedly in treated mice. After some treatments of the animals, the droplets are joined to each other or are fused to

Fig. 1.—White mouse, 45 days old, untreated, given intravenous injection with ferrocyanide. Transverse section of muscular part of diaphragm prepared by freezing and drying directly after the end of the injection and tested for ferrocyanide as Prussian blue stain. Minute droplets of Prussian blue (0.25μ-0.50μ) occur in clusters in the extracellular connective tissue. No ferrocyanide is visible in the muscle fibers. This is an example of the low range of ferrocyanide visible after the dose and time interval used in all experiments; × 1,200.



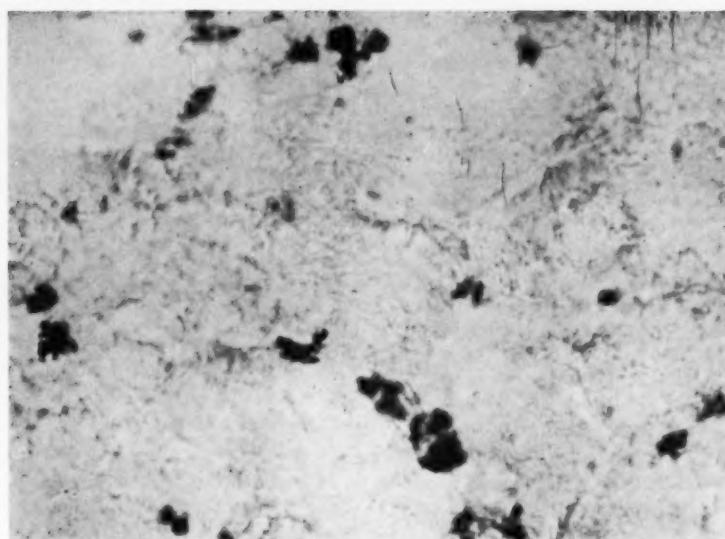


Fig. 2.—White mouse, 45 days old, untreated; injection and preparation as for Figure 1. The droplets are more numerous than in Figure 1. Fine focusing with the microscope resolves the larger droplets into a cluster of smaller-sized ones. This is an example of the high range of ferrocyanide visible under constant experimental conditions;  $\times 1,200$ .

*Effect of Age and Various Treatments of Mice and Rats on Morphological Distribution of Ferrocyanide in Ground Substance of Connective Tissue of Diaphragmatic Muscle\**

Age or Treatment	Distribution of Ferrocyanide								Figure No.
	Discrete Droplets	Partly Fused	Fused Droplets	Small Pools	Large Pools	Difuse	Less than Normal	More than Normal	
<b>A. Normal, untreated</b>									
1. White mice									
Age, days	4								
	8		+		++	++		+++	
				++	++	++		++	3
	12			++	+	+		++	
	24		+	+	+			++	
	30		+	+	+			++	
	37	+++	+		$\pm$				
	45	++	$\pm$						
	540	$\pm$					++		1,2
									4
2. Hybrid mice									
Age, days	70		++						
	1,095	+++			++	++	+	+++	5
									6
B. Hydration									
C. Dehydration		++							
D. Denervation									
E. Inflammation									
Scarfied peritoneal surface									
Non-scarfied pleural surface	++	$\pm$							8
F. Aminocetomitrile		++							
G. Liothyronine	++								
H. Aminocetomitrile + liothyronine	++	+	++	+					
I. Estrogen									
J. Deoxycorticosterone acetate	+								
K. Cortisone	++								10
L. Ascorbic acid	++	$\pm$							
M. Cortisone + ascorbic acid	++								11
N. Vitamin A	++	$\pm$							
O. Parathyroid hormone					++	++	+	+++	12
P. Rachiogentic diet (rats)						+++	+++	+++	13
Q. Control normal diet (rats)	+	$\pm$							14

\* The relative prominence (+++, ++, +,  $\pm$ ) of droplets of various kinds, pools or large vacuoles, and of diffusely distributed ferrocyanide are given, as compared with the appearance in young adult untreated white mice about 45 days old.

FERROCYANIDE IN GROUND SUBSTANCE

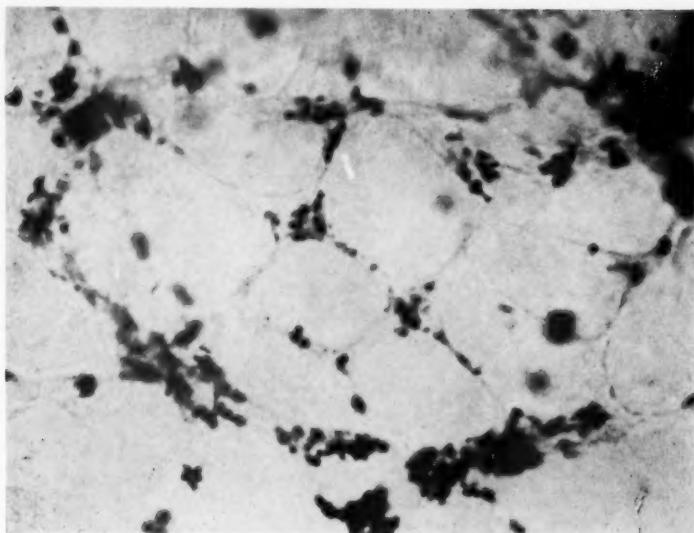
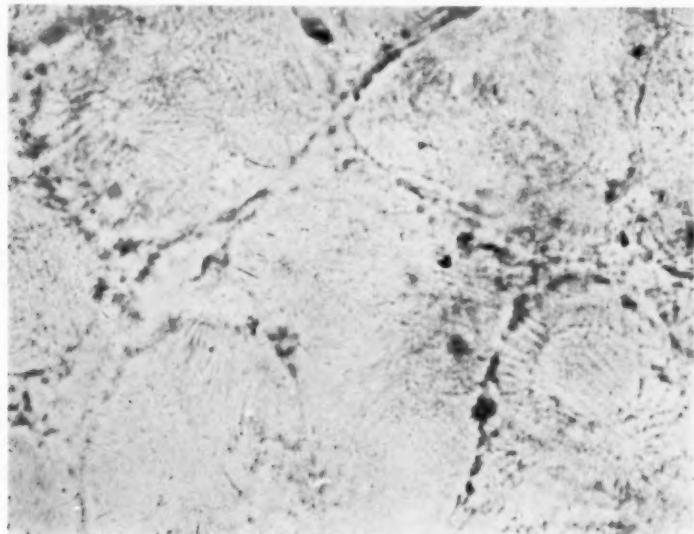


Fig. 3.—White mouse, 45 days old, untreated; injection and preparation as for Figure 1, except that the slow injection was intracardiac. Ferrocyanide is present in larger amounts than in mice 45 days old, and the droplets occur as larger pools which are joined in part by diffusely spread ferrocyanide;  $\times 1,200$ .

form vacuoles or pools of appreciable size or the ferrocyanide may appear as a diffusely, evenly distributed substance with no trace of droplets. Similarly, the num-

ber may be enormously increased or markedly reduced, as compared with the density in the diaphragm of the untreated young adult mouse. The observations have been

Fig. 4.—White mouse, 540 days old, senile, untreated; injection of ferrocyanide and preparation as in Figure 1. A few minute droplets occur in the ground substance—much less than in Figure 1;  $\times 1,200$ .



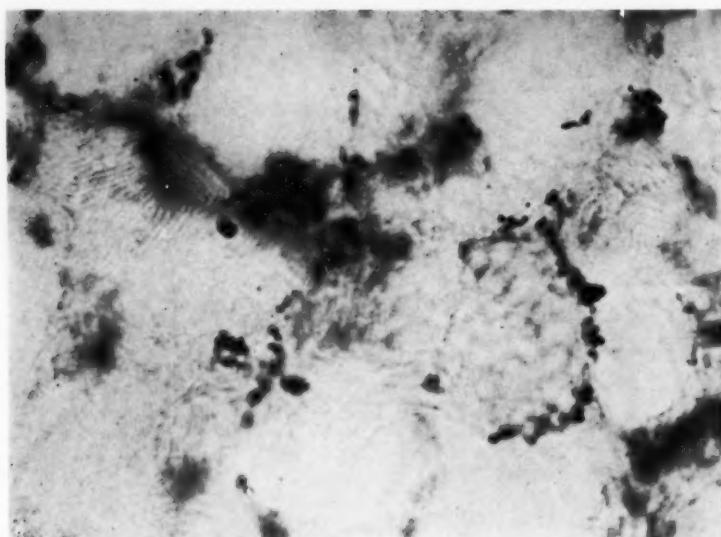
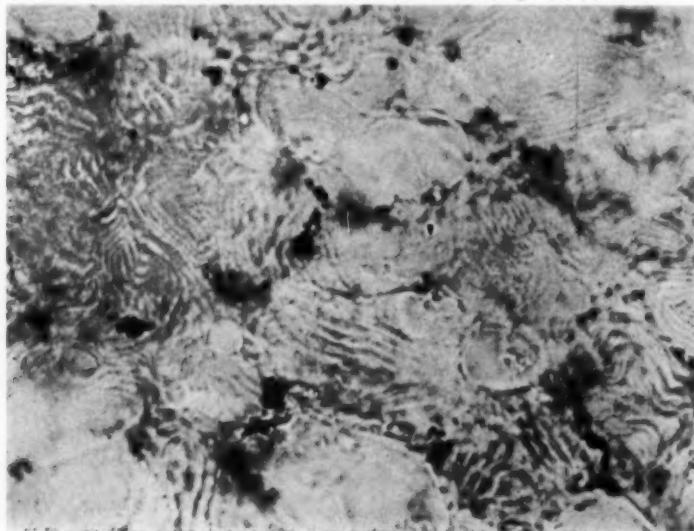


Fig. 5.—Hybrid dark mouse, 70 days old, untreated; injection of ferrocyanide and preparation of diaphragm as for Figure 1. Ferrocyanide is distributed in the ground substance of the connective tissue in a manner more nearly comparable with that in the immature mouse of the white strain (Fig. 3) than with that of the young adult white mouse (Figs. 1 and 2);  $\times 1,200$ .

summarized in tabular form and grouped as numbers according to the treatment of the animals. The observations may be summarized briefly under four headings:

Fig. 6.—Hybrid dark mouse, 1,095 days old but not senile; untreated; injection of ferrocyanide and preparation of diaphragm as for Figure 1. Ferrocyanide is distributed in the ground substance more nearly in the same way as in the diaphragm of the immature or young adult white mouse (Figs. 1-3) than as in the senile white mouse (Fig. 4). Ferrocyanide is, however, more restricted than in the adult of the same strain (Fig. 5);  $\times 1,200$ .

1. During growth of white mice, ferrocyanide is distributed very broadly in the ground substance of the connective tissue. During maturation and going on into old



## FERROCYANIDE IN GROUND SUBSTANCE

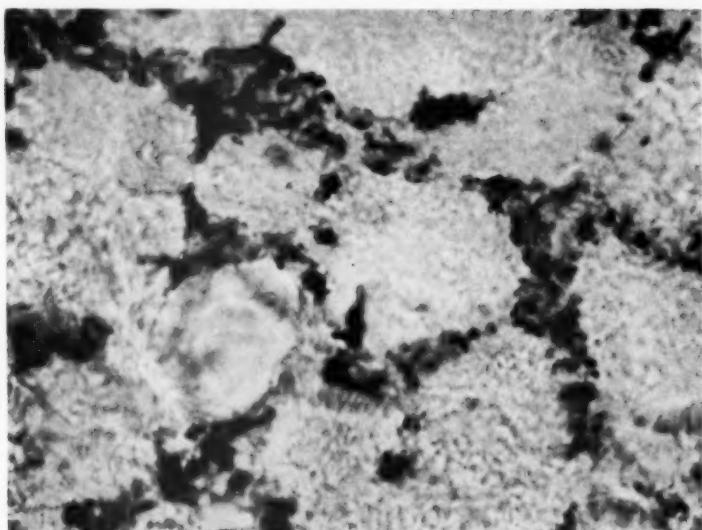
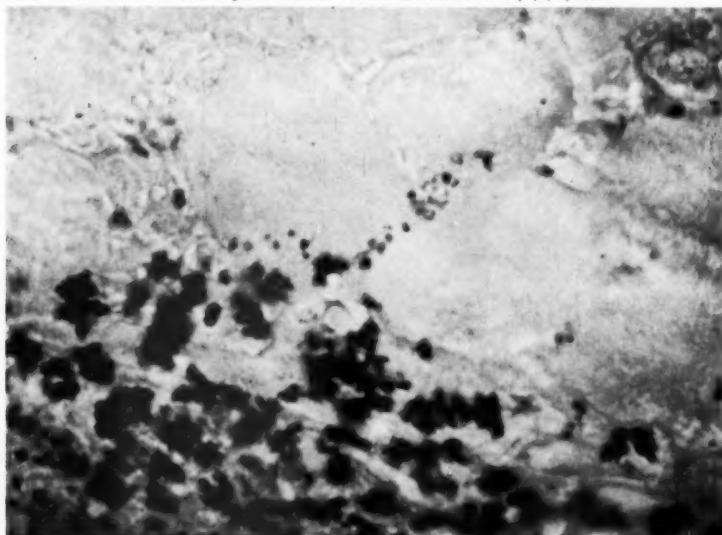


Fig. 7.—Effect of hydration of young adult white mouse on distribution of ferrocyanide in the ground substance of the connective tissue of the diaphragm. The body weight of the mouse had been doubled by intraperitoneal injection of saline. Injection of ferrocyanide and preparation of diaphragm as for Figure 1. There is more ferrocyanide in the ground substance, as compared with untreated controls (Figs. 1 and 2), and the droplets are larger. In addition, there is much diffusely spread ferrocyanide in the ground substance;  $\times 1,200$ .

age, the volume occupied by visible ferrocyanide is progressively restricted and in the oldest animals is limited to minute drop-

lets which are very few in number. The same trend was notable in two age groups of a second, very long-lived strain. Though

Fig. 8.—Effect of early inflammation on the distribution of ferrocyanide in the mouse diaphragm. The abdominal surface of the diaphragm was traumatized, and two days later ferrocyanide was injected and the diaphragm prepared as for Figure 1. The abdominal portion of the diaphragm contains large droplets of ferrocyanide, compared to the thoracic portion, where the distribution of ferrocyanide falls within normal limits;  $\times 1,200$ .



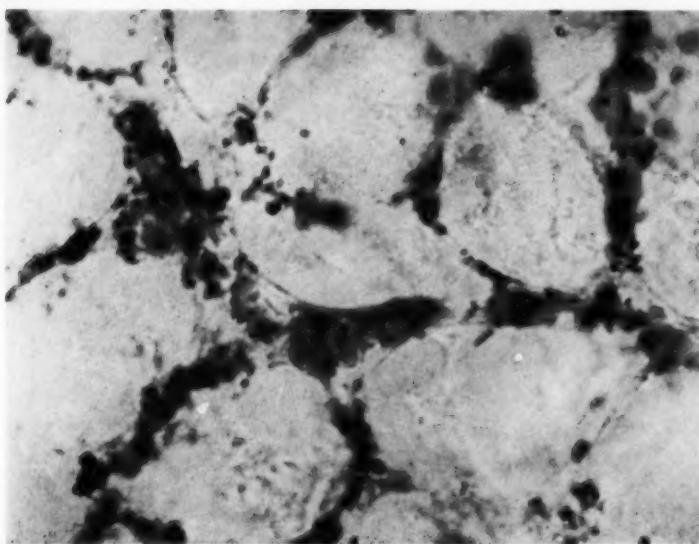


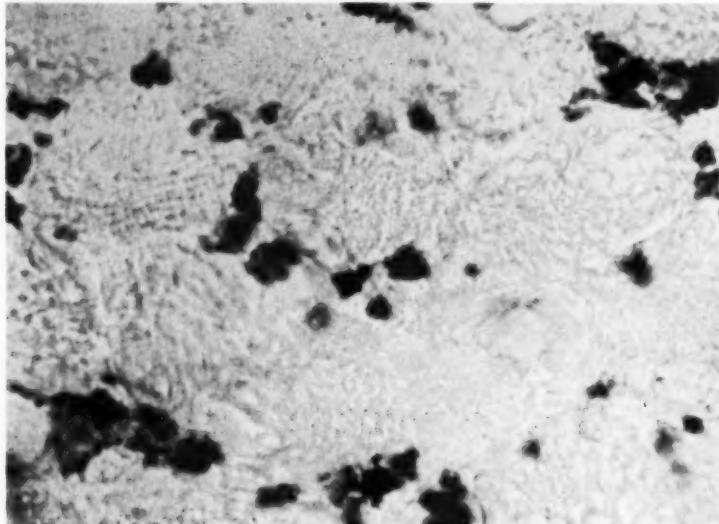
Fig. 9.—Effect of administration of aminoacetonitrile on the distribution of ferrocyanide in the ground substance of the diaphragm of the young adult mouse. As compared with the untreated control preparations (Figs. 1 and 2), ferrocyanide is distributed in the ground substance in the form of small and large pools, with an additional diffuse component;  $\times 1,200$ .

the mice of the second strain were twice as old as the white mice, they behaved and appeared younger than the latter. The ferrocyanide distribution in the ground substance of the diaphragmatic connective

tissue of the 3-year-old mice appeared more like that of immature white mice (Figs. 1-6).

2. Visible ferrocyanide is increased in amount, and the visible droplets are fused or enlarged into pools or even diffusely

Fig. 10.—Effect of administration of cortisone on the distribution of ferrocyanide in the ground substance of the diaphragm of the young adult mouse. As compared with untreated controls (Figs. 1 and 2), ferrocyanide is more broadly distributed in the form of larger pools supplemented by a diffuse component;  $\times 1,200$ .



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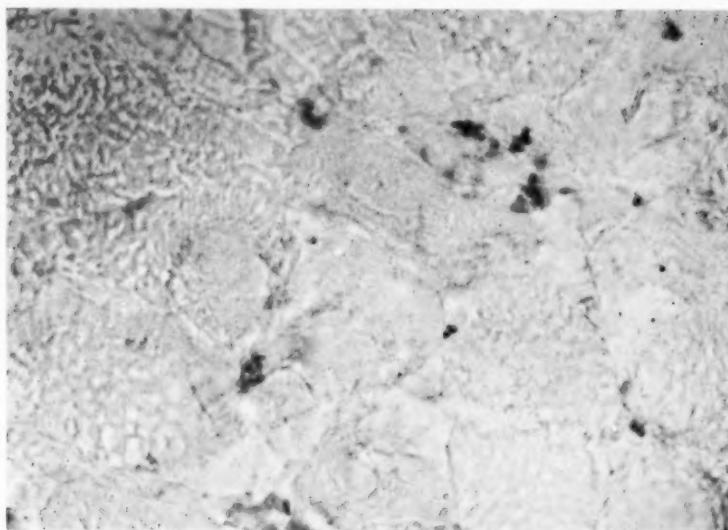
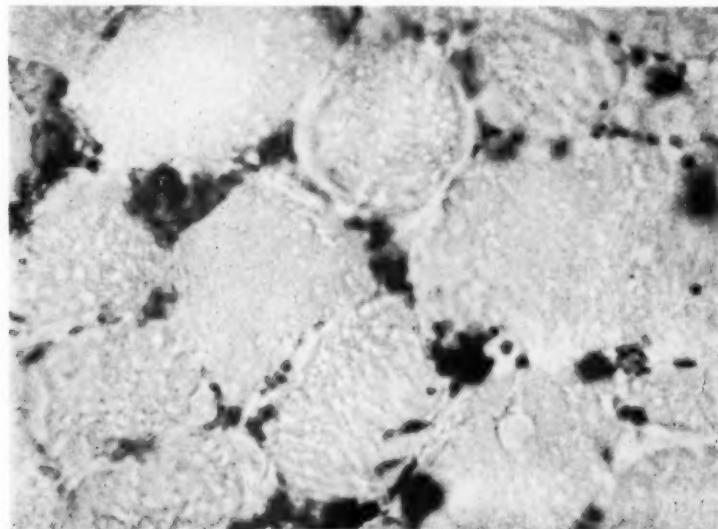


Fig. 11.—Effect of simultaneous administration of cortisone and ascorbic acid on the distribution of ferrocyanide in the ground substance of the connective tissue of the young adult mouse diaphragm. Ferrocyanide is distributed more nearly as in the untreated controls (Figs. 1 and 2), and is in marked contrast with the picture after cortisone alone (Fig. 10). It is interesting that after administration of the vitamin alone the distribution of ferrocyanide is the same as in normal untreated controls;  $\times 1,200$ .

distributed in a number of experimental conditions: hydration (Fig. 7); dehydration (Fig. 8); denervation, inflammation (Fig. 9) and following treatment of the mice with estrogen, deoxy-

lathyrysm following the administration of aminoacetonitrile (Fig. 10) and following treatment of the mice with estrogen, deoxy-

Fig. 12.—Effect of administration of parathyroid extract on the distribution of ferrocyanide in the ground substance of the connective tissue of the diaphragm of the young adult mouse. Ferrocyanide occurs as large pools with an abundant diffusely distributed component, as compared with the more restricted disposition of ferrocyanide in untreated controls (Figs. 1 and 2);  $\times 1,200$ .



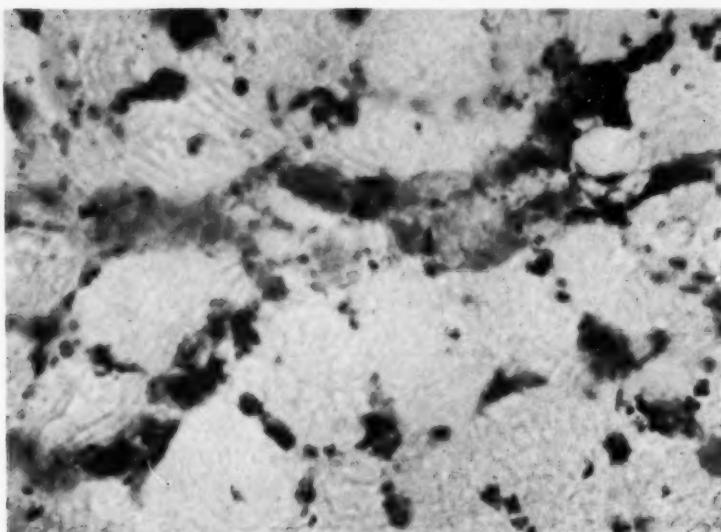


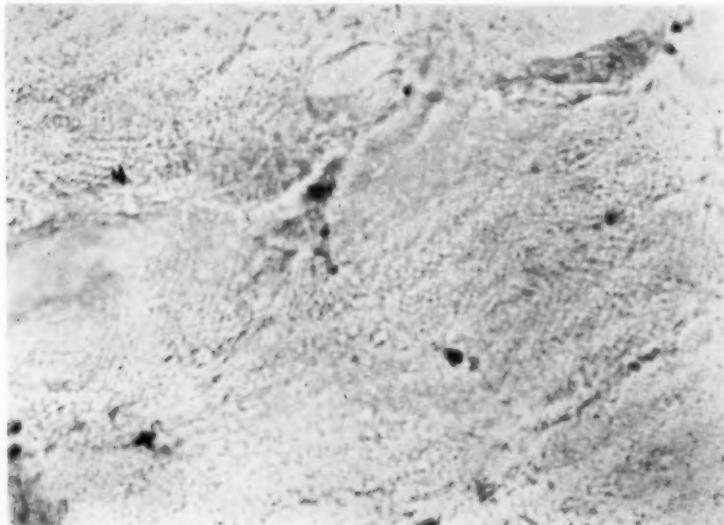
Fig. 13.—Effect of rachitogenic diet on the distribution of ferrocyanide in the ground substance of the connective tissue of the diaphragm of the young rat. Ferrocyanide was injected intraperitoneally, and the diaphragm was prepared as for all preceding animals. Extensive large pools and diffusely distributed ferrocyanide occur in the ground substance. Compare with Figure 14,  $\times 1,200$ .

corticosterone acetate, cortisone (Fig. 10), and parathyroid hormone (Fig. 12). In rachitic rats, the same condition was observed in the ground substance of the con-

nective tissue of the diaphragm (Figs. 13 and 14).

3. The distribution of visible ferrocyanide is not influenced by the administration

Fig. 14.—Control rats fed a normal diet and given injections of ferrocyanide, as for Figure 13. Ferrocyanide is sparsely distributed, chiefly as discrete and partly fused droplets. No large pools or diffuse components are present;  $\times 1,200$ .



FERROCYANIDE IN GROUND SUBSTANCE

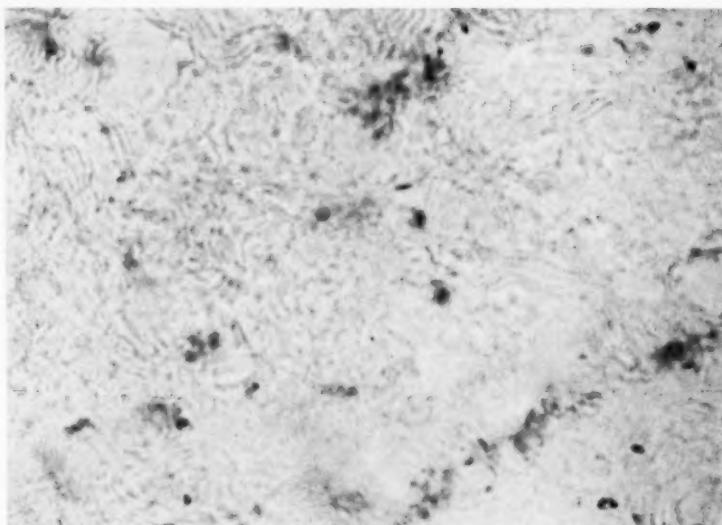


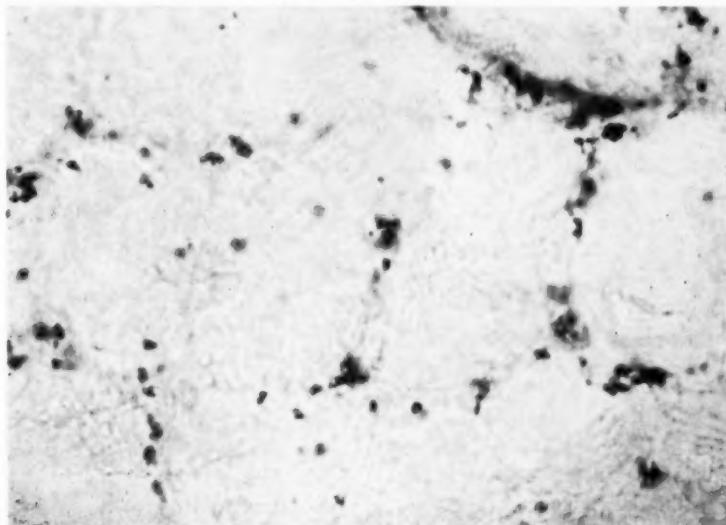
Fig. 15.—*In vitro* preparation of intact mouse diaphragm incubated in Krebs-Ringer-ferrocyanide solution for 10 minutes. The diaphragm was frozen and dried, and sections were tested for ferrocyanide as Prussian blue. Ferrocyanide is visible as discrete droplets like those in the ground substance of the *in vivo* preparations of the diaphragm of the normal, untreated mouse;  $\times 1,200$ .

to normal mice of liothyronine, ascorbic acid, or vitamin A.

4. The increase in amount of visible ferrocyanide and the altered distribution in two of the conditions noted above (2) are

reduced or prevented by the simultaneous administration of certain reagents which are, however, ineffective when given alone to otherwise untreated normal mice (3, above). The effects on ferrocyanide dis-

Fig. 16.—*In vitro* preparation of intact mouse diaphragm, prepared as for Figure 15 but incubated for 45 minutes. There is more ferrocyanide in the ground substance in the form of fused droplets, together with a diffuse component;  $\times 1,200$ .



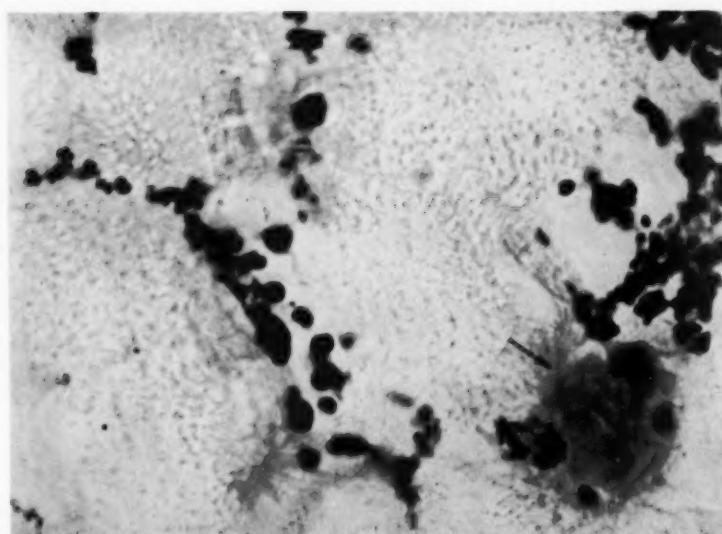


Fig. 17.—In vitro preparation of intact mouse diaphragm, prepared as for Figures 15 and 16 but incubated for 120 minutes. Ferrocyanide is present as large pools. Some muscle fibers are damaged and contain ferrocyanide (arrow);  $\times 1,200$ .

tribution associated with treatment with aminoacetonitrile are partially neutralized by the simultaneous administration with liothyronine, and the marked effect of cortisone is abolished by simultaneous administration of ascorbic acid (Fig. 11).

*In Vitro.*—The in vitro observations on the isolated diaphragm preparations may be summarized more simply. After 10 minutes of incubation in Krebs-Ringer-ferrocyanide solution of the intact mouse diaphragm, ferrocyanide is visible in a few sparse very fine droplets, with an extremely delicate diffusely distributed background limited exclusively to the connective tissue (Fig. 15). Ten minutes later the droplets appear as clusters. After 30 minutes of incubation, the discrete droplets and clusters of droplets containing ferrocyanide have formed chains and are otherwise partly joined and fused and the diffusely distributed ferrocyanide is more apparent. At 45 minutes, the droplets have fused (Fig. 16). These are more extensive at one hour and have formed larger pools with much diffusely distributed ferrocyanide. At two hours (Fig. 17), the large pools are to some

extent continuous. Although up to this time no ferrocyanide was visible in the muscle fibers, at this time some muscle fibers appear blue. In a single preparation incubated for seven hours, a larger number of fibers contain ferrocyanide. The distribution of visible ferrocyanide in the intact rat diaphragm incubated for one hour corresponds with that in the similar mouse preparation.

Visible ferrocyanide is distributed very differently when the muscle fibers have been cut. The blue color appears from the *first* within the muscle fibers and penetrates them progressively in time. The rate of penetration is about  $30\mu$  per minute. The visible ferrocyanide in the region closer to the central tendon—that is, at the end of the muscle where the muscle fibers are intact—is confined as in uncut mouse diaphragm preparations to the ground substance of the connective tissue.

#### Comment

As in the work of Chase, ferrocyanide is distributed at least partly in the water-rich phase of the ground substance of the

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connective tissue of the muscular portion of the diaphragm. The water-rich phase was considered to be in equilibrium with the denser, water-poor phase of the ground substance, and it was considered that both phases were subject to change. The experiments and observations recorded in this paper disclose that the equilibrium in the two phases represented by the distribution of visible ferrocyanide is very sensitive to experimental treatment of the animal. The magnitude of the changes visible with the light microscope under varied conditions is very striking. An attempt will be made to relate these whenever possible to pertinent findings in the literature.

*Changes with Age.*—The progressive changes in the distribution of ferrocyanide with a series of increasingly older white mice indicate that the extensive water-rich phase of very young becomes progressively restricted. This may perhaps be related to the finding by Boas and Foley (1954)<sup>2</sup> that there is more water in the skin of young rats than in older ones. A more significant finding is that ground substance stainable by the periodic acid-leukofuchsin method is more water-soluble in embryonic and young rats than in older rats (Gersh and Catchpole).<sup>3</sup> It is also significant that although the extracellular volume of distribution of inulin and ferrocyanide of infants is 30%-42% of the total body weight (Calcagno, Husson, and Rubini, 1951)<sup>4</sup> the corresponding value in the adult human is 15%-16% (Gaudino, Schwartz, and Levitt, 1948).<sup>5</sup>

It is perhaps significant that the visible ferrocyanide of the ground substance of a hybrid strain of mice with prolonged longevity is more extensive than in the shorter-lived white mice. The older group of the former looked and acted younger than the oldest group of the latter. This association of physiological aging with a progressive loss of the water-rich phase of the ground substance of connective tissue should be worked over more extensively statistically and quantitatively with a larger series of strains of mice having widely differing normal age spans.

*Effects of Hydration, Dehydration, and Inflammation.*—In all three conditions, visible ferrocyanide in the ground substance is increased. Instead of the restricted distribution of the water-rich phase of the normal mouse, this phase appears as enlarged pools which become confluent. In these animals it may be inferred that the water-rich phase of the ground substance of muscle is enlarged at the expense of the water-poor phase. It may also be inferred that in acute inflammation the water-rich phase is affected like that in hydrated animals. The mechanism for the change following dehydration is not obvious.

*Denervation of the Diaphragm.*—This is followed by an increase in visible ferrocyanide and by its segregation in part in coarser vacuoles and its appearance in diffuse form. Hines and Knowlton (1937)<sup>6</sup> showed that an increase in total water takes place in denervated muscles, and Eichelberger, Akeson, and Roma (1957)<sup>7</sup> confined this increase largely to the extracellular mass. The increase in the size of the droplets and in the amount of visible ferrocyanide in the ground substance of the connective tissue thus localizes the water increment after denervation still more closely to the water-rich phase of the ground substance. The mechanism responsible for the change is not known.

*Lathyrism.*—Ponseti et al. (1956),<sup>8</sup> Selye (1957),<sup>9</sup> and Bachhuber et al. (1955)<sup>10</sup> were all able to duplicate symptoms of lathyrism by administering certain nitrile compounds. The symptoms include widening and slipping of epiphyses, disruption of tendons and ligaments, degenerative arthritis, and dissecting aneurysms. Ponseti (1954)<sup>11</sup> postulated that the nitrile causes a change in the ground substance and that this underlies the symptoms caused by the drug. The work reported above shows that feeding with aminoacetonitrile results in an increase in the water-rich phase of the ground substance of the connective tissue. This change, by itself, would not seem to account for the symptoms of lathyrism, for similar changes, sometimes even more ex-

tensive, do not result in the syndrome. One must assume, then, that some other mechanism is involved. It is suggested that the collagen itself has been depolymerized and that the weakening of the collagen fibers leads to the development of lathyrism. Selye (1957)<sup>12</sup> found that thyroid administration could prevent the appearance of these symptoms, and Ponseti (1957)<sup>13</sup> showed that liothyronine was even more effective. The changes in the water-rich phase of the ground substance are partly prevented by the administration of *L*-triiodotyrosine in the dose used, although the drug administered alone to normal animals does not seem to influence the ground substance. The mechanism of action of either drug is unknown.

*Estrogen, Deoxycorticosterone Acetate, and Cortisone.*—Estrogen results in the retention of body water in the monkey (Krohn and Zuckerman, 1937),<sup>14</sup> more particularly in muscle (Zuckerman, Palmer, and Hanson, 1950).<sup>15</sup> A similar water retention was described also in the dog by Thorn and Harrop (1937)<sup>16</sup> and by Thorn and Engel (1938).<sup>17</sup> The observations reported in the preceding section localize the site of water retention even more closely, at least in part, to the water-rich phase of the ground substance of the connective tissue of muscle.

Zuckerman, Palmer, and Hanson (1950)<sup>15</sup> showed also that administration of deoxycorticosterone acetate results in a similar retention of water in muscle. This finding is consistent with the increased sodium space resulting from the administration of deoxycorticosterone acetate, reported by Gaudino and Levitt (1949)<sup>18</sup> and Gamble (1953).<sup>19</sup> In a study of experimental wound healing, Pirani, Stepto, and Sutherland (1951)<sup>20</sup> found that after the administration of deoxycorticosterone acetate the connective tissue stains more metachromatically and is more highly stained by the periodic acid-leukofuchsin method, as compared with untreated controls. In the light of the increase in the amount of visible ferrocyanide and the altered distribution

observed after the administration of deoxycorticosterone acetate it is suggested that the excess water is retained at least in part in the water-rich phase of the ground substance.

Numerous papers attest to the profound effect of cortisone on connective tissue, especially in inflammation. Only two will be mentioned here, as they bear particularly on the ground substance. Castor and Baker (1950)<sup>21</sup> showed that local treatment with cortisone results in increased stainability of the ground substance with the periodic acid-leukofuchsin method. Joseph, Engel, and Catchpole (1954)<sup>22</sup> showed that the administration of cortisone results in the formation of prominent ice crystals in the ground substance of the connective tissue of the skin. The latter finding indicates that more water is present in the ground substance after treatment with cortisone than in untreated controls. In mice treated with cortisone visible ferrocyanide is increased and is present largely in a diffuse (nonvacuolar) form. This increase in the water-rich phase would tend to furnish the basis for the prominence of ice crystals after the freezing and drying of large specimens. It is suggested that with enlargement of the aqueous phase of the ground substance more periodic acid-Schiff (PAS)-positive material is disaggregated and is more readily amenable to staining by this method.

*Vitamin A and Ascorbic Acid.*—The administration to untreated mice of these vitamins in the doses employed had no notable effect on the distribution or amount of ferrocyanide in the ground substance. This is in agreement with the similar conclusion of Halasz and Marx (1932),<sup>23</sup> Lauber, Nafziger, and Bersin (1937),<sup>24</sup> Bourne (1942),<sup>25</sup> and others that excess ascorbic acid does not accelerate the healing of fractures and of Rodahl (1950)<sup>26</sup> that only enormous doses of vitamin A, much larger than employed in this work, produce significant lesions in mice. The striking effect of simultaneous administration of ascorbic acid and cortisone on the distribu-

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tion of ferrocyanide was unexpected, and the result is at present unexplained. To what extent treatment with ascorbic acid would protect against the effects of other hormonal substances is not known.

**Parathyroid Hormone.**—One report bears particularly on the findings reported in the preceding section. Engel, Joseph, and Catchpole (1954)<sup>27</sup> showed that the administration of parathyroid extract results in a decrease in the electronegative, relatively immobile, colloid of the ground substance of the connective tissue of the gingivae, skin, bone, and muscle. The decrease in the colloidal charge was attributed by Engel to an increase in the water-rich phase of the ground substance. This is in full accord with the increase in the water-rich phase, as reflected in the markedly altered distribution of visible ferrocyanide after treatment of the mice with parathyroid extract. From this it may be inferred that the "target organ" of parathyroid extract is not limited only to bone but is more generalized; the "target organ" of parathyroid extract may constitute the ground substance of the connective tissue throughout the body. The mechanism of action on ground substance of the parathyroid is unknown.

**Rickets.**—Visible ferrocyanide in the ground substance of the connective tissue in diaphragmatic muscle is notably increased in amount and is more extensively distributed in the form of large pools and in a diffuse state. This finding is interpreted as indicating that the aqueous phase of the ground substance of muscle is enlarged at the expense of the water-poor phase. The effects of the high-calcium, low-phosphorus, vitamin D-depleted diet are not only apparent on cartilage and calcification of bone but would also seem to be more general, perhaps influencing the ground substance of the connective tissue throughout the body.

**In Vitro Experiments.**—The mechanism whereby hormonal and other factors influence the relative proportions of water-rich (colloid-poor) and water-poor (colloid-rich) ground substance is unknown. The

first question, it would seem, would be whether these substances act directly on the ground substance of the connective tissue or whether they influence the connective tissue cells to modify the ground substance. The *in vitro* preparations are intended to serve in elucidating this question. At least for a certain time of incubation, the distribution of ferrocyanide resembles that in the diaphragm of mice given injections *in vivo* with ferrocyanide. It is hoped in this way, by the use of a series of selective cellular enzyme inhibitors during or preceding incubation in ferrocyanide, to learn whether cellular activity is necessary for the maintenance of the two phases of ground substance and perhaps which activities are involved.

It should be emphasized that visible ferrocyanide is absent from muscle fibers, except when they are injured (as after prolonged incubation) or cut. This fact has an important bearing on the interpretation of much of the research on permeability of muscle (of diaphragm) to a wide variety of substances. This subject is discussed in great detail by Hechter.<sup>30</sup>

**General Comments.**—In this paper, data are presented which supplement those of Chase that the ground substance of connective tissues is organized at the submicroscopic level as a two-phase system in equilibrium. The relative proportions of the two phases may be influenced by numerous factors. From theoretic considerations based on the electrochemical behavior of connective tissue, Joseph, Catchpole, and Engel<sup>22,27,28</sup> had predicted the existence of these phases, and the present paper, like those of Bondareff<sup>29</sup> and of Chase, serves to define morphologically their nature and extent. An important contribution of the electrometric studies is that the relative proportions of the two phases determine automatically the relative proportion of certain ions in the ground substance. This implies that every change in the distribution of visible ferrocyanide described is accompanied by a change in the ionic content of the ground substance or, in other words, of

the ionic environment of the cells of the connective tissue, as well as of the parenchymatous cells of various tissues and organs. The exact nature of the ionic changes accompanying the morphological variations in the water-rich phase of the ground substance would depend on quantitative values of colloidal charge in the submicroscopic units. It is, of course, not possible now to study these small structures individually, and only the over-all changes in ground substance are amenable to study. From the electrometric studies it may be predicted that all the hormonal substances studied should result in a decreased total calcium and magnesium concentration and a decreased sodium- and potassium-ion concentration in the ground substance which constitutes the cellular environment.

The results reported in this paper reinforce and expand the conclusion of Gersh and Catchpole that hormones influence the state of the ground substance of connective tissues. These authors based their conclusion in part on changes in the solubility of those components of the ground substance which are stainable with the periodic acid-leukofuchsin method. It is suggested that the increased solubility of the PAS-positive components of the ground substance may reflect at least in part the relative increase in the water-rich phase. The ferrocyanide method appears to be a more sensitive method in quantitative terms as well as in terms of dimension.

### Summary

A great variety of treatments influences the distribution of ferrocyanide in the ground substance of the connective tissue of diaphragmatic muscle. These include hydration, dehydration, denervation, inflammation, and administration of aminoacetonitrile and various hormonal substances, such as liothyronine (*I*-triiodothyronine), estrogen, deoxycorticosterone acetate, cortisone, parathyroid hormone (Parathormone), and ascorbic acid (vitamin C). Prominent changes take place also in rachitic animals fed a high-calcium, low-phosphorus, vita-

min D-depleted diet. Marked changes develop during growth and aging of mice. The organization of the ground substance of the connective tissue as a two-phase system in equilibrium seems to be very responsive to numerous factors. If visible ferrocyanide is considered to be confined to the water-rich phase it is clear that the relative proportion of this phase, as compared with the water-poor phase, is altered. The theoretical significance of these findings is discussed.

This work was supported by a grant from the Commonwealth Fund and the Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago. Dr. Isidore Gersh assisted in this study. Dr. Eric L. Simmons supplied the hybrid mice used in these experiments and Dr. William Bondareff supplied the Swiss albino mice. Abbott Laboratories supplied the aminoacetonitrile hydrogen sulfate.

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# Structure of Basement Membranes of Fat Cells

WILLIAM H. CHASE, M.D., Chicago

The intimate relations of basement membrane and reticular fibers have not been clarified. On the basis of preparations studied with the light microscope, reticular fibers have been described lying beneath the basement membrane, enclosed within the basement membrane, or constituting the basement membrane. The contributions of the principal exponents of these various views have been outlined by Lillie.<sup>1</sup> On the basis of thinner preparations studied with the electron microscope, basement membranes have been described by many as homogeneous structures, with only occasionally some evidence of internal structure. The staining of the basement membranes and of reticular fibers is not selective in these preparations, and it has been difficult to delineate the intimate relations of reticular fibers, collagen fibrils, and basement membrane.

In the course of a study of fat tissue, it was unexpectedly found that basement membranes and collagen fibrils were clearly stained in preparations studied with the electron microscope. Since adipose tissue is a site rich in reticular fibers<sup>2</sup> and as a basement membrane has been described around fat cells,<sup>3</sup> it seemed worth while to study their relations in detail.

It is clear from this study that single collagen fibrils or small bundles of them are included within the basement membrane, where they are surrounded by an otherwise homogeneous component. These

Submitted for publication Aug. 28, 1958.

From the Department of Anatomy, The University of Chicago. Fellow of the Helen Hay Whitney Foundation.

The expenses for this study were defrayed by grants from the Clara A. and Wallace C. Abbott Memorial Research Fund of The University of Chicago, the Helen Hay Whitney Memorial Research Fund, and the Commonwealth Fund.

fibrils are continuous with those which comprise the reticular fibers lying in the intercellular space.

## Experimental

Ten adult Sprague-Dawley rats were used in this study. In order to reduce the size of the fat cells the animals were made lean by feeding a restricted diet.<sup>4</sup> Some were allowed to eat a mixture of olive oil and flour 1-24 hours before use. Under ether anesthesia the fat pad in the inguinal region was exposed by careful dissection. The adipose tissue was grasped and lifted between two forceps, so as to form a thin sheet, and frozen by the pouring on of liquid propane, cooled to about -175°C. To avoid warming of the tissue, liquid nitrogen was immediately poured over the area and the entire animal was then quickly plunged into liquid nitrogen. The frozen fat was chipped off under liquid nitrogen with bone forceps.

Small fragments of tissue were dried in vacuo at about -37°C by use of the apparatus described by Finck.<sup>5</sup> After 12-24 hours the drying tube was placed in an ice-brine bath at -6°C. When the tube reached this temperature the fixative solution, which was also kept cold, was run in and the vacuum broken. The fixative consisted of 1% OsO<sub>4</sub> dissolved in 1 M CaCl<sub>2</sub> at -6°C. At the end of one hour the tissues were transferred to Feigl tubes while still in cold fixative and allowed to warm to room temperature over a period of about 15 minutes. They were then washed rapidly in three changes of distilled water, dehydrated through one-half-hour changes in graded alcohols, and permitted to harden overnight in 95% alcohol. A celloidin-methacrylate imbedding procedure was used.

## Results

The large size of the tissue predisposes to ice-crystal formation during the freezing of the specimen. The artifacts associated with growth of ice crystals become prominent at a distance of 20μ-30μ or more from the surface. The slow penetration of the fixative solution may also result in failure of preservation of the fat vacuoles in the

## BASEMENT MEMBRANES OF FAT CELLS

center of the tissue block. For these reasons, observations were confined to the superficial parts, although it was found that basement membranes were well preserved even in the presence of gross distortion of adjacent cytoplasm by ice crystals.

It should be mentioned that sectioning was often difficult owing to inadequate hardening of the tissue and/or poor infiltration with methacrylate. Generally, the fat at the margins of the block would cut smoothly, but elsewhere marked displacements frequently occurred.

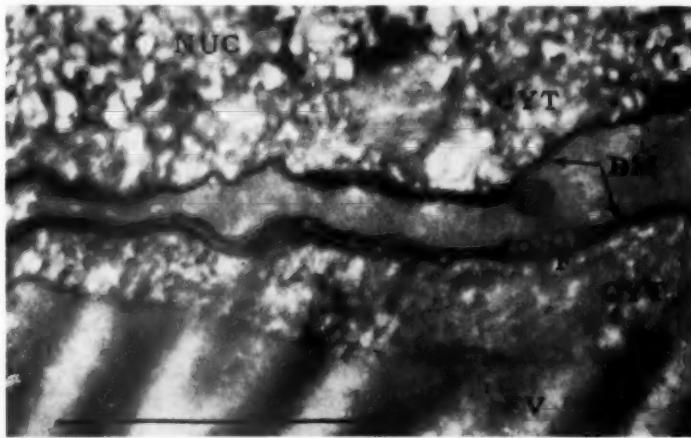
The basement membrane of fat cells appeared in sections as a homogeneous or finely granular, intensely osmophilic line varying in thickness from 100 to 500 Å. (Fig. 1). The boundaries of this line were sharp. Adjacent basement membranes occasionally appeared to fuse, but this may have been due to overlay. More frequently they were separated by an irregular space containing homogeneous less dense material. Spaces containing only methacrylate were frequently seen between basement membranes and were probably artifacts resulting from incomplete infiltration of

displacements during sectioning. Capillary endothelial basement membranes were stained in a manner similar to that of the fat cells.

While the basement membrane often appeared to lie directly on the fat-cell cytoplasm, in many areas it was separated from the cytoplasm by a structureless space measuring about 50 Å. and having low density. Adjacent to this, a less well-defined layer of similar dimension and moderate density merged with the cytoplasm.

Reticular fibrils were sharply outlined and stained (Fig. 4) but were poorly visualized if calcium chloride was omitted from the fixative solution. The fibrils were very numerous adjacent to blood vessels, where they formed bundles up to 0.5 μ thick. Elsewhere they frequently occurred singly, or several were gathered together to form small bundles or straps. The fibrils were bordered by a thin, dark line. Cross striations were clearly seen, and adjacent fibrils often had their striations in register (Fig. 6). Dark and light bands of about equal size alternated along the length of the fibrils. The dark bands contained four symmetrical

Fig. 1.—Section of rat fat, frozen-dried and postfixed in  $\text{OsO}_4$  solution containing 1 M  $\text{CaCl}_2$ . Parts of two fat cells (*CYT*) are shown bordered by densely stained basement membranes (*BM*). At *F*, a number of collagen fibrils lying within the basement membrane are sectioned transversely. They are surrounded by the densely stained homogeneous component of the basement membrane. Elsewhere, collagen fibrils showing typical periodicity are seen in longitudinal section within the basement membrane. Part of a fat vacuole (*FI'*) is seen below, while a portion of a fat-cell nucleus (*NUC*) is present at the upper left. Bar at lower left indicates 1 μ.



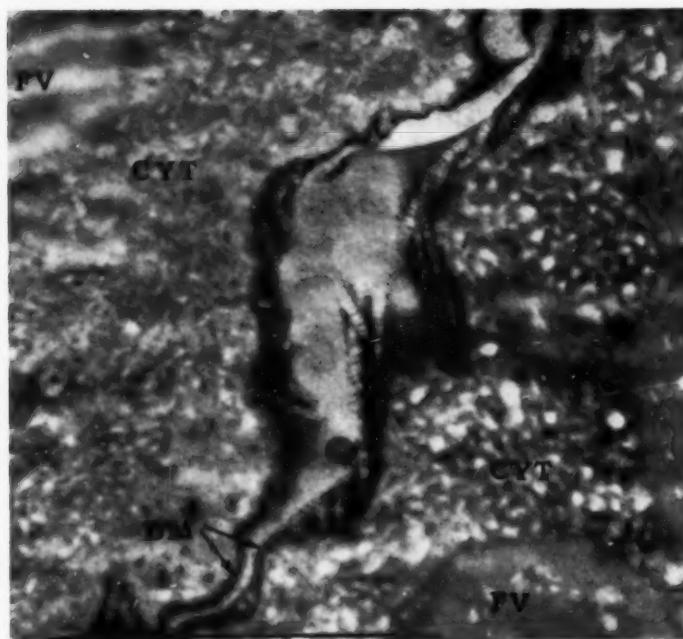


Fig. 2.—Electron micrograph of rat fat, prepared as in Figure 1. Portions of two fat cells, including cytoplasm (CYT) and fat vacuoles (PV), are bounded by densely stained basement membrane (BM), which appears obliquely sectioned at the center of the photograph. The disposition of the cross striated collagen fibrils within the basement membrane is clearly evident. Bar at bottom indicates  $1\mu$ .

transverse dense lines separated by three lighter lines, while in the light band three transverse light lines were separated by

two narrow dense lines. Thus, 12 intra-period bands could be distinguished. The width of the major period varied from 310

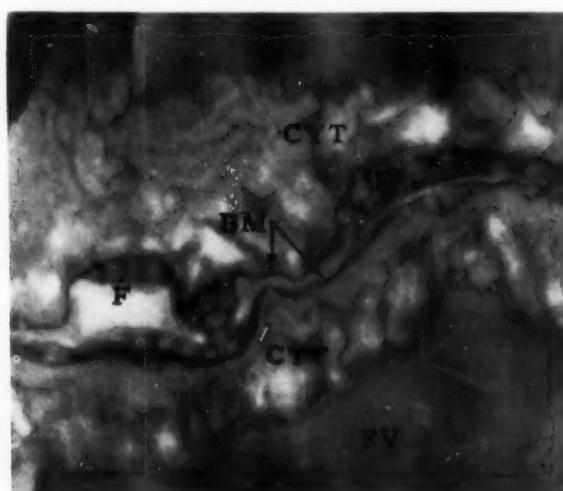


Fig. 3.—Electron micrograph of rat fat, prepared as in Figure 1. Adjacent basement membranes (BM) bounding two fat cells (CYT) containing fat vacuoles (PV) are shown. Numerous collagen fibrils (F) lying in the basement membranes have been sectioned transversely and are seen to be partly or entirely surrounded by the homogeneous component of the basement membrane. Part of the longitudinally sectioned fibril is also present. The cytoplasm of the fat cells is grossly distorted by ice crystals. Bar at bottom left is  $0.1\mu$ .

## BASEMENT MEMBRANES OF FAT CELLS

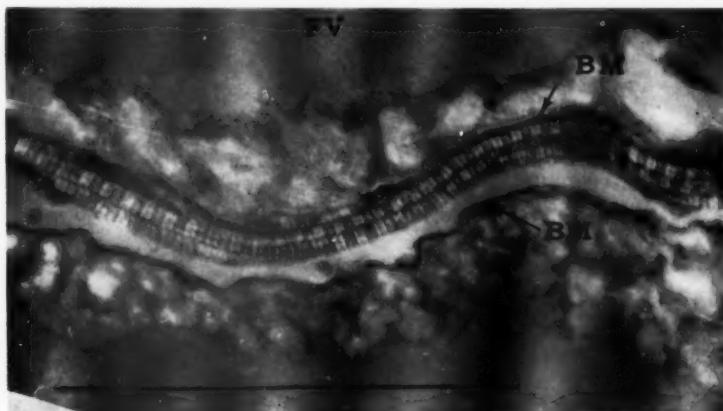


Fig. 4.—Electron micrograph of rat fat, showing similar aspects as in Figure 3. A bundle of collagen fibrils, comprising a reticular fiber, lies in the intercellular space, where it appears in longitudinal section. Bar at bottom indicates  $1\mu$ .

to 700 Å. in different photographs. The average was 530 Å. The diameter of the fibrils varied between about 200 and 500 Å.

The relationship of the collagen fibrils to the basement membrane was most interesting. In sections cut longitudinal to the

fiber axis, single fibrils or small bundles were present both outside of and within the basement membrane. In the latter case they were enveloped by the homogeneous component of the basement membrane (Figs. 1-3). Occasionally fibrils were seen enter-

Fig. 5.—Section of rat fat, prepared as in Figure 2. A portion of a fat cell (FC) with its fat vacuole (FV) lies below. Part of a capillary (CAP) is seen above. Both the capillary and the fat cell have a densely stained basement membrane (BM). In the intercellular space small bundles of collagen fibrils (F) are seen, comprising reticulin fibers. These have a well-defined cross striation. Bar at bottom left is  $1\mu$ .

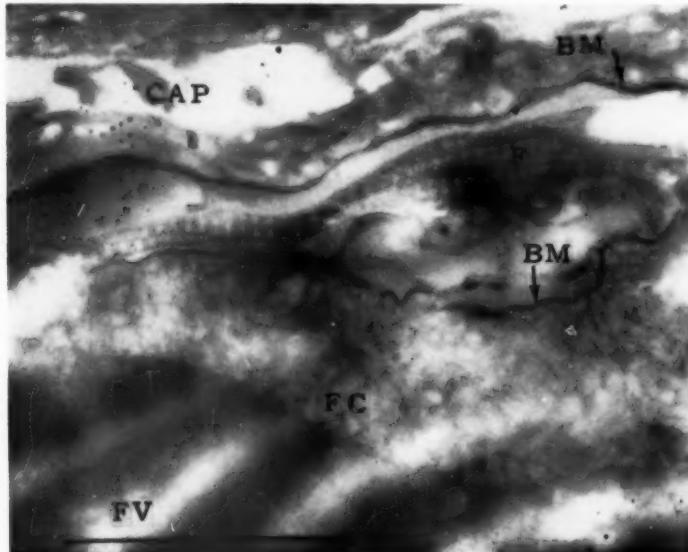




Fig. 6.—High-power electron micrograph of loosely arranged collagen fibers found living between two fat cells. The tissue was prepared as in Figure 1. The dark and light bands forming the major period are clearly evident. Twelve intraperiod bands can be distinguished. Bar at bottom left is  $0.1\mu$ .

ing the basement membrane from the intercellular space. Within the basement membrane, the fibrils often rested side by side, thus forming straps. When sections were cut perpendicular to the fibril axis, the latter appeared as negative images surrounded by the denser basement membrane (Fig. 3). This gave a fenestrated appearance to the basement membrane.

The structure of the fat cell will be described in a subsequent paper.

#### Comment

There is evidence that the basement membrane has two components, one fibrous in nature, the other homogeneous. The technique described in this report appears to delineate sharply the two components, so that their intimate relationship becomes clear. The characteristic periodicity identifies the reticular fibers which comprise a variable number of collagen fibrils. The average major period spacing of  $530\text{ \AA}$ . is similar to that reported by Jakus<sup>6</sup> in Descemet's membrane. The dark and light

bands may correspond to those described by Schmitt,<sup>7</sup> which he labeled A and B, respectively. Isolated fibrils and probably also small bundles, continuous with the reticular fibrils, enter and course within the basement membrane, where they are enveloped by the homogeneous component of the latter.

Examination by others of tissues fixed by immersion in aqueous osmium tetroxide solutions has occasionally revealed a laminated or fibrillar structure of the basement membrane.<sup>8-17</sup> The collagen fibrils have been poorly defined in such studies, however, appearing to be obscured by the homogeneous component of the basement membrane. Kramer and Little,<sup>18</sup> in a study of isolated renal reticulin, have shown that the latter may exist as membranes in which are imbedded collagen fibrils. The relationship of these structures to the basement membranes shown in this report is not clear.

The reason for the intense osmophilia of the basement membrane in the preparations is not known and is in contrast with the

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appearance of the basement membrane of tissues fixed by immersion in  $\text{OsO}_4$  solutions without freeze-drying. The role which calcium chloride plays in rendering the reticular fibers visible is also unknown. The procedure has been applied to other tissues but without success.

### Summary

Adipose tissue of rats was prepared for electron microscopy by freezing and drying and post fixation with aqueous  $\text{OsO}_4$  solution containing calcium chloride. The sections showed an intense staining of basement membranes in this tissue, while the presence of the calcium salt caused reticular fibers to be clearly outlined and stained. Collagen fibrils were enclosed within the homogeneous component of the basement membrane and were continuous with those which comprise the reticular fibers lying between adjacent basement membranes.

Department of Anatomy, The University of Chicago (37).

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# The Arthus Phenomenon in the Colon of Rabbits

*A Serial Histological Study*

MOSHE B. GOLDGRABER, M.D., and JOSEPH B. KIRSNER, M.D., Chicago  
With the Technical Assistance of Jean Ablaia, B.A., and Harold Ford, B.S.

## Introduction

The Arthus phenomenon, originally described in the skin of rabbits in 1903, is an expression of local anaphylaxis and results from repeated injections of antigen. The clinical counterpart of this phenomenon has been reported frequently in man,<sup>2-13</sup> especially when antitoxic sera were in common use. The Arthus reaction has been observed in the experimental animal in organs other than the skin,<sup>14</sup> and it is safe to assume that such reactions may occur in the gastrointestinal tract of man.<sup>15</sup> The histopathology of the Arthus reaction, first described by Arthus and Bréton,<sup>16</sup> has been dealt with subsequently by Gerlach,<sup>17</sup> Opie,<sup>18</sup> Laporte,<sup>19</sup> and Pagel.<sup>20</sup> These observations concerned early phases of the response, and the findings varied when a prolonged detailed study was attempted.<sup>20</sup> The Arthus reaction in the colon has not been investigated previously. It seemed desirable, therefore, to study this phenomenon in the colon of rabbits, with observations for periods up to four weeks.

## Material and Method

Thirty white New Zealand rabbits on a pellet diet and water ad lib. were sensitized to crystalline egg albumen according to three separate schedules, A, B, and C (Tables 1, 2, and 3).

A. Fifteen rabbits received 1 cc. of a 2% solution of egg albumen\* on three days in three suc-

Submitted for publication Oct. 10, 1958.

Department of Medicine, The University of Chicago.

This study was supported in part by grants from the National Institute of Health and the Wallach Fund for Research in Gastrointestinal Diseases (The University of Chicago).

\* Emulsol Flake Egg Albumen, from the Emulsol Egg Products Corporation, 59 E. Madison St., Chicago.

cessive weeks in five different portals: intravenous, intraperitoneal, intradermal, intramuscular, and subcutaneous. The first injection consisted of 1 cc. of Freund's<sup>21</sup> adjuvant, 1 cc. of isotonic saline, and 100 mg. of egg albumen.

B. Nine animals received highly purified albumen† through multiple portals, as in Schedule A, but without the adjuvant.

C. Six animals were sensitized as in Schedule B. The eliciting injection was into multiple sites in the colon.

Two groups of animals were used as controls. In one group, nonsensitized rabbits received an injection of 0.2 cc. of 0.5% egg albumen subserosally. The second group was sensitized with egg albumen via the multiple portal method; the challenging injection consisted of 0.2 cc. of saline subserosally into the colon.

All animals were operated upon, and 0.2 cc. of 0.5% solution of egg albumen was injected subserosally into the proximal and distal colon, except for the last six rabbits in whom multiple sites of the colon were injected. A black nonabsorbable surgical (silk) suture in the adjacent mesocolon indicated the site of the expected reaction. The animals of Group A were killed at the following hours postoperatively: 6, 12, 24, 48, 72, 96, 120, 144, and 168. The animals of Group B were killed at 120, 144, and 168 hours and 2, 3, and 4 weeks, postoperatively. The six animals of the multiple-site group were killed at 24 and 168 hours after operation. In all animals, after a lethal dose of barbiturate, the abdomen was reopened; the sites of injection were carefully inspected, and the gross findings were noted. Tissues were fixed in buffered formalin; blocks in paraffin were cut, and sections were stained, mostly with hematoxylin and eosin.

## Comment

The primary purpose of this paper was to study the later developments of the

† Pentex, five times crystallized albumen from (Biochemical) Pentex (service). P. O. B. 248, Kankakee, Ill. Composition of each preparation verified by electrophoretic measurements.

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Fig. 1 (Female rabbit No. 56). — Photomicrograph 12 hours after eliciting injection. Spectacular edema with separation of capillary vessels. Reduced 10% from mag.  $\times 460$ .

Arthus reaction; therefore, the serial examination was initiated at six hours after the eliciting injection. At that time there was a fully developed pattern of tissue changes, characterized by edema, cellular infiltration

at the bases of crypts and around vessels, hemorrhage, and necrosis of the muscularis propria. For the sake of clarity, the features of this reaction are considered individually.

Fig. 2 (Male rabbit No. 15). — Photomicrograph 24 hours after eliciting injection, deeply hemorrhagic area.



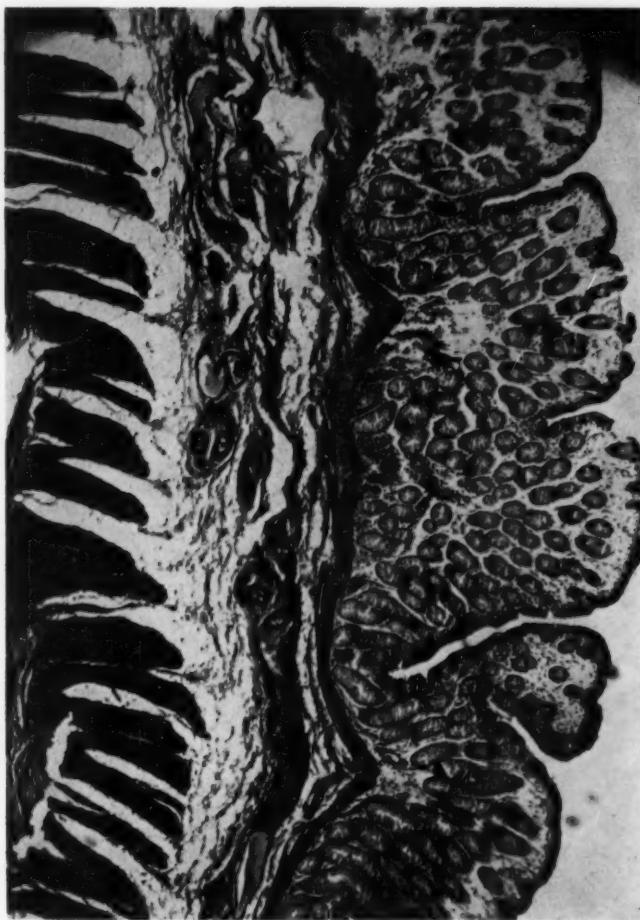


Fig. 3 (Male rabbit No. 15). — Photomicrograph 24 hours after eliciting injection. Note lymphatic dilatation and perivascular infiltrates. Reduced 10% from mag.  $\times 60$ .

**Edema.**—This feature was noted throughout the observation period. It was more spectacular at the beginning, distending capillaries and the lamina propria with fluid. Edema was described by Arthus and Bréton and mentioned by many other investigators.<sup>17-20,22</sup> It was observed in the allergic reactions involving the human gastrointestinal tract.<sup>25,26</sup> Edema was thought to be a result of stasis observed directly at the microscope by Fröhlich.<sup>27</sup> Manwaring et al.<sup>28</sup> regarded the changes in the intraluminal pressure during anaphylactic reactions as the reason for interference with the circulation, exudation of fluid beneath the epithelium, secondary rupture of the latter

with ulceration, and hemorrhage. Manwaring et al.<sup>29</sup> also found a 75% reduction in the rate of perfusion of sensitized lungs when an antigen solution was used. Increased capillary permeability was thought to be the dominant physiologic reaction in allergy. Whatever the mechanism, edema is an important component of the Arthus phenomenon and was defined as one of the cardinal three features of the reaction as early as 1909 by Thompson and Marchildon.<sup>30</sup>

**Cellular Infiltration.**—Leukocytes were present in only moderate numbers in the first 24 hours. Later, leukocytes were sparse and careful search was required to

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Fig. 4 (Male rabbit No. 15). — Photomicrograph 24 hours after eliciting injection. Hemorrhage in the submucosa, partially replaced by collections of mononuclears and leukocytes. The muscularis mucosae is broken, and the mucosa is thinned. Reduced 10% from mag.  $\times 105$ .



demonstrate their presence. This finding is in accord with those of Dienes and Mallory,<sup>31</sup> who observed a small percentage of polymorphonuclears if a small amount of antigen was injected into sensitized animals. Large doses of antigen provoke more exudation and necrosis; 0.2 cc. of antigen in a 0.5% solution is a small quantity; but perhaps it reproduces more clearly the changes in naturally occurring disease, presumably involving contact of relatively small doses of antigen with hypersensitive tissues. The prevalent elements of infiltration in the lamina propria and around vessels, therefore, were lymphocytes, plasma cells, and, to less degree, eosinophils. Plas-

ma cells appeared early in the present material but never attained proportions to merit a separate phase of plasma-cell maturation, as described by Gell and Hinde.<sup>32,33</sup>

*Vascular Damage.*—Thrombosis was observed in a lymphatic only once, in an animal killed 24 hours after the eliciting injection. Vascular thrombosis is a more frequent feature of the Shwartzman phenomenon of the colon.<sup>34</sup> Hyaline degeneration of the arteriolar wall was observed occasionally. Perivascular cellular infiltration was more frequent than true vasculitis.

*Hemorrhage.*—This is a prominent feature of the Arthus phenomenon, demonstrable as early as 24 hours and subsequently



Fig. 5 (Male rabbit No. 15). — Photomicrograph 24 hours after eliciting injection. Large hemorrhage in muscularis; edema, lymphatic dilatation, and cellular infiltration in submucosa. Reduced 10% from mag.  $\times 100$ .

throughout the observation period. It may exist without obvious damage of the vascular wall and may be absent despite hyaline degeneration of the vascular wall. The hemorrhage may be located in the submucosa or in the muscularis; however, it is less pronounced and less frequent than in the Shwartzman phenomenon.<sup>34</sup>

*Damage of Smooth Muscle.*—Necrosis of the muscularis propria was observed at six hours. This, therefore, is a feature of the acute, immediate type of hypersensitivity reaction; necrosis also has been observed in the Shwartzman phenomenon.<sup>34</sup> The muscle fibers seem fragmented and assume a deep purple color with hematoxylin and

eosin. Later, apparent initially in the three-day specimens, another type of lesion appears—thinning and disappearance of the external layer of the muscularis propria after the cells have been compressed by deposition of collagen between the fibers. This feature, defined as collagenosis of external muscle layer, has been observed repeatedly in animals treated according to Schedules A and B. In vitro responses of intestinal smooth muscle were obtained when strips were tested against highly diluted solutions of specific antigen.<sup>35</sup> It is of interest that Domagk<sup>36</sup> considered smooth muscle the main target of anaphylactic reactions.

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Fig. 6.—(Male rabbit No. 594).—Photomicrograph 30 hours after eliciting injection. Arteriole with hyaline degeneration of wall and perivascular accumulation of cells, predominantly eosinophils;  $\times 300$ .

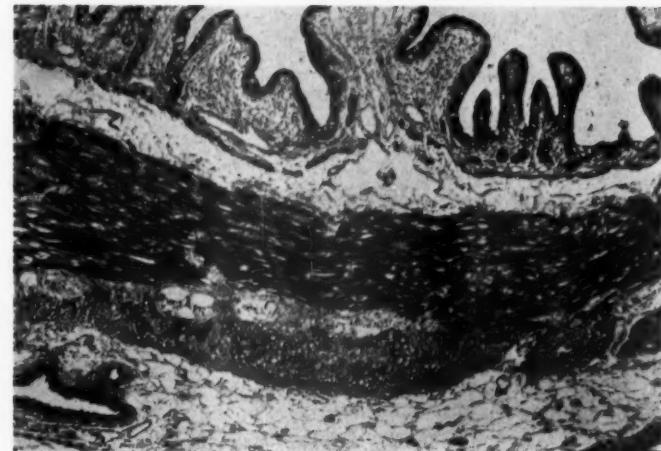
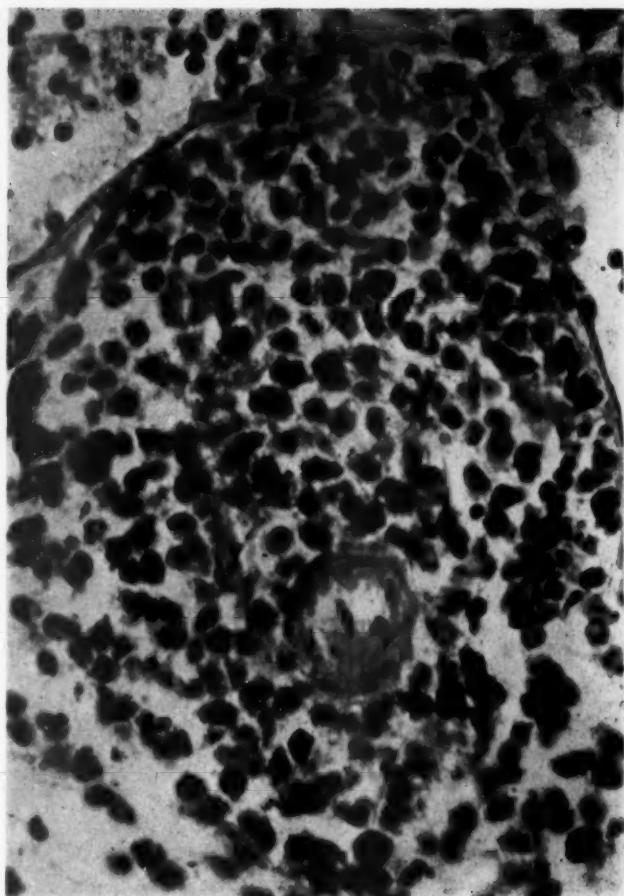


Fig. 7 (Female rabbit No. 65).—Photomicrograph 72 hours after eliciting injection. Degeneration of muscle fibers in the external layer of the muscularis propria, with deposits of collagen in the interstices; collagenesis. Reduced 35% from mag.  $\times 100$ .



Fig. 8 (Female rabbit No. 63). — Photomicrograph 72 hours after eliciting injection. Acute vasculitis as evidenced by degeneration of arteriolar wall, invaded by leukocytes. Pronounced perivascular infiltration of monocytes and leukocytes. Reduced 35% from mag.  $\times 100$ .

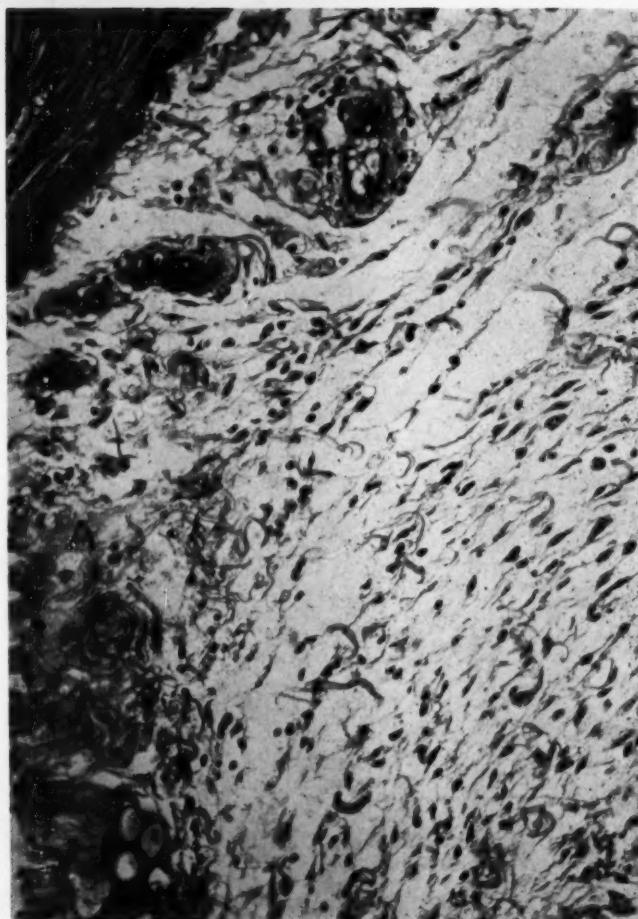


Fig. 9 (Male rabbit No. 25). — Photomicrograph 96 hours after eliciting injection. External muscle layer (*A*) contains only residual cells. Diffuse histiocytosis of serosa. Reduced 10% from mag.  $\times 250$ .

ARTHUS PHENOMENON

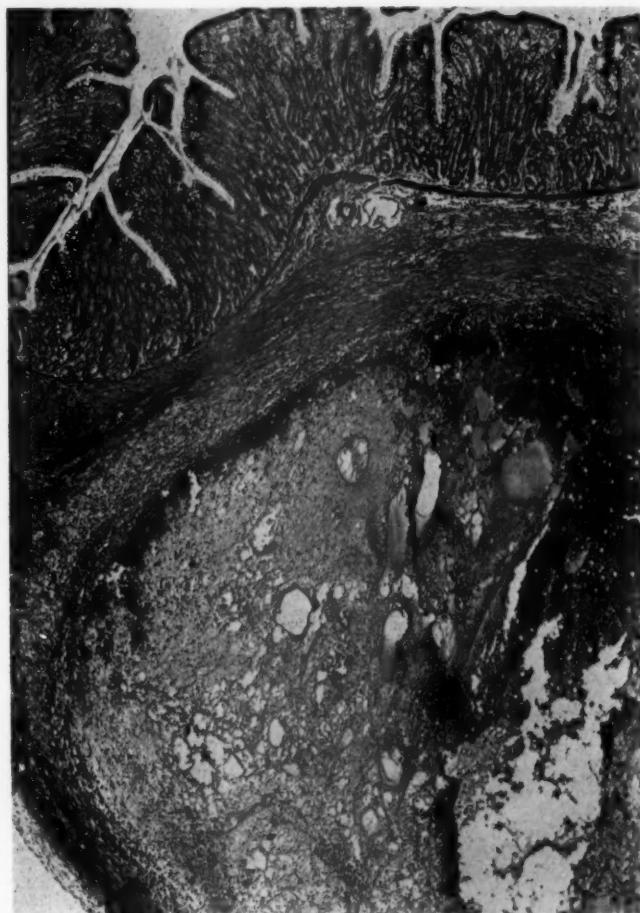


Fig. 10 (Female rabbit No. 61). — Photomicrograph 120 hours after eliciting injection. Large focus of hemorrhage, surrounded by a fibroblastic and cellular wall. Reduced about 10% from mag.  $\times 48$ .

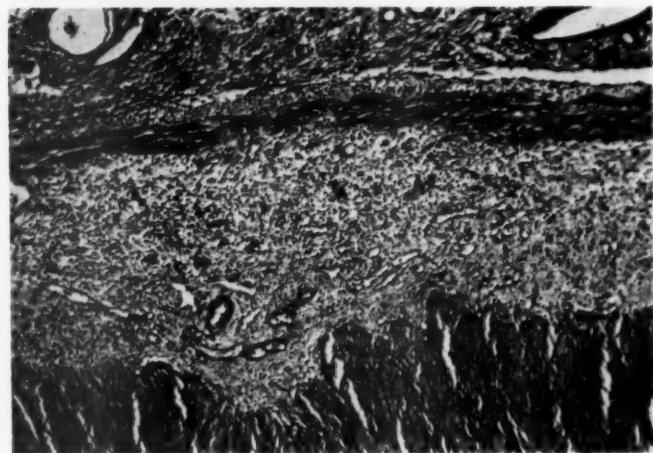


Fig. 11 (Female rabbit No. 61). — Photomicrograph 120 hours after eliciting injection. Cellular-vascular tissue, with pronounced eosinophilia above and below the muscularis mucosae. Reduced about 35% from mag.  $\times 115$ .



Fig. 12 (Female rabbit No. 24). — Photomicrograph 144 hours after eliciting injection. Granulomata and giant cells. Darkly stained necrotic tissue. Reduced 10% from mag.  $\times 250$ .

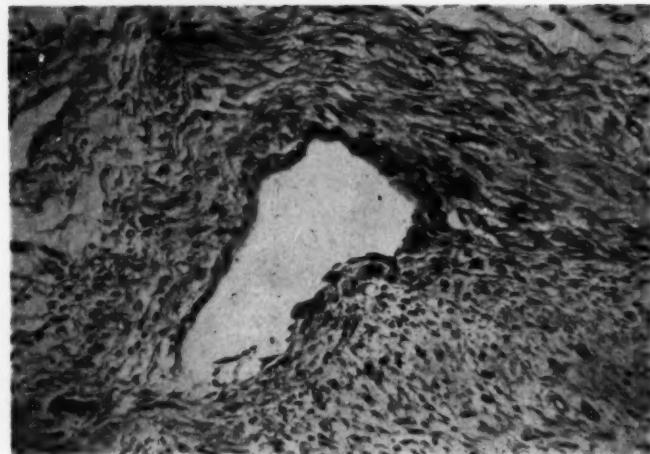


Fig. 13 (Female rabbit No. 24). — Photomicrograph 144 hours after eliciting injection. Dilated lymphatic with endothelial proliferation. Reduced 35% from mag.  $\times 250$ .

ARTHUS PHENOMENON

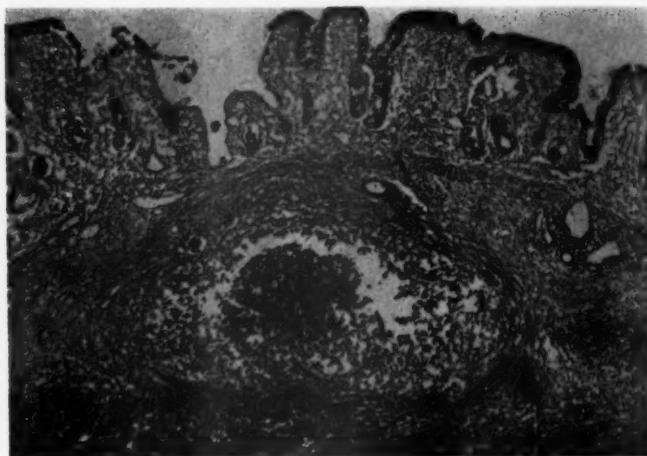


Fig. 14 (Female rabbit No. 64). — Photomicrograph 168 hours after eliciting injection. Focus of hemorrhage, with little surrounding fibroblastic activity. Reduced 35% from mag.  $\times 75$ .



Fig. 15 (Female rabbit No. 170). — Photomicrograph seven days after eliciting injection. There are ulceration, hemorrhage, and diffuse and localized eosinophilia. Reduced 10% from mag.  $\times 75$ .

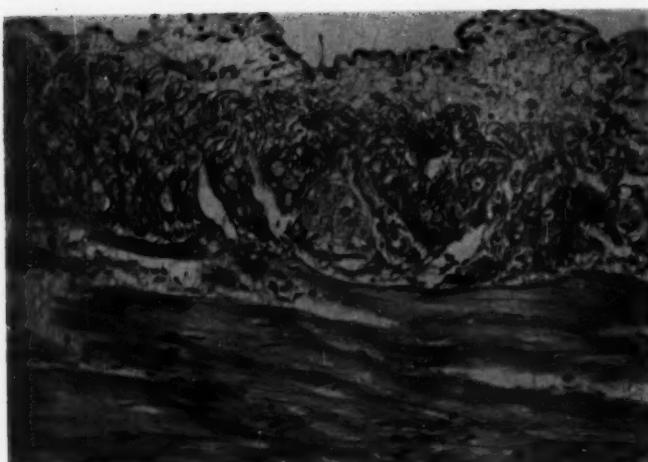


Fig. 16 (Male rabbit No. 170).—Photomicrograph seven days after eliciting injection. Collagen fibers are deposited between the muscle cells of the external layer of the muscularis propria. Reduced 35% from mag.  $\times 310$ .

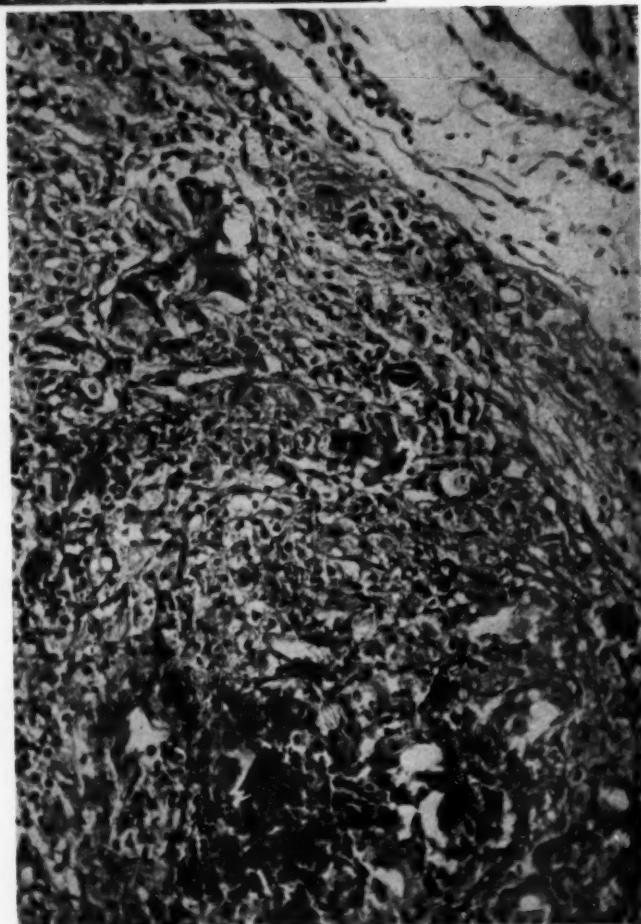


Fig. 17 (Female rabbit No. 142).—Photomicrograph seven days after eliciting injection. Granulomatous involvement of the serosa with giant cells. Reduced 10% from mag.  $\times 175$ .

## ARTHUS PHENOMENON

TABLE I.—Schedule A—Gross and Microscopic Findings

Animal, No.	Sex	Hour	Reaction *	Gross Description	Microscopic Findings
54	F	6	+++	Hyperemia & hemorrhage with blister formation in both proximal & distal colon	Edema of mucosa & submucosa; cellular infiltration at bases of crypts perivascular infiltrates; necrosis of muscularis; fully developed reaction
56	F	12	++	Slight hyperemia in terminal colon; fecal impaction at site of injection in proximal colon	Spectacular edema with distention of capillaries (Fig. 1); vascular engorgement and round-cell infiltration in submucosa (Fig. 3); focal accumulations of leukocytes
15	M	24	++++	Deeply hemorrhagic area with dark blister formation in proximal colon (Fig. 2)	Multiple foci of hemorrhage (Fig. 4) in submucosa & muscularis (Fig. 5), some of them replaced by mononuclears and leukocytes
53	M	24	++	Slight hyperemia in both proximal & distal colon	Edema & perivascular accumulations of perithelial & endothelial origin; large cellular collections in muscularis propria
62	F	24	++	Hyperemia, hemorrhage, & blister in proximal & distal colon	Edema & increased cellularity in lamina propria; perivascular infiltrates (Fig. 3) in submucosa; thrombosis of lymphatics; hemorrhage & infiltration of serosa
504	M	30	+++	Pronounced hyperemia, hemorrhage, & dark blister; reaction extended for 1 in.	Cellular infiltration & hemorrhage in serosa; perivascular infiltrations; eosinophilic cell accumulation around arteriole with hyaline degeneration of wall (Fig. 6)
52	F	48	++	Slight hyperemia	Increased cellularity of lamina propria; thickening & infiltration in the serosa; edema in the submucosa
586	F	48	++	Slight hyperemia with hemorrhage at 1 site (terminal colon)	Diffuse cellular infiltration in lamina propria; marked edema & dilation of lymphatics in submucosa
65	F	72	—	Negative	Thinning & disappearance of external layer of muscularis propria; focal degeneration of muscle fibers with deposit of collagen between spaces occupied by muscle cells (Fig. 7)
63	F	72	—	Negative	Edema of submucosa; marked vasculitis & perivasculitis in serosa & muscularis (Fig. 8)
25	M	96	—	Negative	Diffuse histiocytosis of serosa with blood vessels manifesting pericytic proliferation; degeneration & disappearance of external muscle layer (Fig. 9); increased cellularity in lamina propria
21	M	96	+	One small hemorrhage in the terminal colon 1 mm. in diameter	Edema & cellular infiltration in lamina propria; edema & perivasculitis in the submucosa; thickening of serosa & perivasculitis in mesocolon
61	F	120	++	Nodule in proximal colon, slight hyperemia in terminal site	Large hemorrhagic focus with fibrocellular wall (Fig. 10); large submucosa with cellulovascular tissue rich in eosinophiles (Fig. 11); similar cells in lamina propria
24	F	144	++	Hemorrhage in proximal colon, 3×3 mm.; no reaction in terminal colon	Thickening of mesocolon with histiocytosis & fatty tissue giant cells; collagenosis & atrophy of external layer of muscularis propria; granulomata & giant cells (Fig. 12); proliferation of lymphatic endothelium (Fig. 13); minimal hemorrhage
64	F	168	—	Negative	Hemorrhage with little fibroblastic activity in submucosa (Fig. 14); collagenosis of external muscle layer; perivascular infiltrates in submucosa & mesocolon

\* + indicates very slight reaction; ++, slight reaction; +++, moderate reaction, and +++, severe reaction.

TABLE 2.—Schedule B—Gross and Microscopic Findings

Animal, No.	Sex	Time from Eliciting Injection	Reaction	Gross Findings	Microscopic Findings
167	F	120 hr.	—	Negative	Local thickening of serosa with infiltration of round cells; large cellular infiltrate in muscularis & submucosa; collagenosis of external muscle layer with localized areas of atrophy
169	M	144 hr.	++	Small white nodule in proximal colon	Local thickening of serosa with cellulovascular tissue, including a focus of old hemorrhage; edema & cellular infiltration of lamina propria; pronounced eosinophilia
170	M	168 hr.	++	Small white nodule on proximal colon	Ulceration & hemorrhage in mucosa (Fig. 15); diffuse & localized eosinophilia as in eosinophilic granuloma; collagenosis of external muscle layer (Fig. 16)
163	F	2 wk.	—	Negative	Infiltration of lamina propria; thickening & fibrosis of serosa; collagenosis of muscularis propria in external layer
165	M	2 wk.	—	Negative	Diffuse marked infiltration of round cells in lamina propria; collagenosis of external muscle layer
173	F	3 wk.	—	Negative	Edema of mucosa & increased cellularity at base of crypts; thickening of serosa & downgrowth of muscle fibers of external muscle layer
164	F	3 wk.	—	Negative	Increased cellularity of base of crypts & localized collections of lymphocytes in the lamina propria; thickened serosa & collagenosis of external layers of muscularis propria
166	F	4 wk.	—	Negative	Mild increase of cellularity in lamina propria
171	F	4 wk.	++	Slight hyperemia of terminal colon	Pronounced increase in round-cell infiltration diffusely & in dense collections; external muscularis with collagenosis

**Granuloma Formation With or Without Giant Cells.**—These formations, overlooked by earlier investigators, were described first by Opie,<sup>18</sup> in the local hypersensitive state. Others<sup>19,20,37</sup> observed their formation at various intervals, ranging from three days to three weeks after the eliciting injection. Goddard<sup>38</sup> emphasized the granuloma and giant cell as a "qualitative" distinctive feature of the allergic reaction. Others<sup>39,40</sup> have confirmed this "qualitative" difference

between the normal and the hypersensitive state. The so-called foreign-body giant cell also seems to have an allergic basis.<sup>41</sup> Granulomata and giant cells were observed repeatedly in the present material six and seven days after the eliciting injection.

**Tissue Eosinophilia.**—Rössle<sup>42</sup> described eosinophilia in tissues repeatedly injected with the same antigen. Eosinophilia was not recorded by Arthus and Bréton<sup>16</sup> and by other investigators.<sup>17,18,31</sup> Berger and

TABLE 3.—Schedule C—Gross and Microscopic Findings

Animal, No.	Sex	Hour	Reaction	Gross Findings	Microscopic Findings
139	F	24	+++	Mild hyperemia in 4 of 5 sites in terminal colon	Proximal colon: cellular infiltration in lamina propria; dilated lymphatics & hemorrhage in submucosa Distal colon: edema, cellular infiltration at base of crypts; perivascular infiltrates
132	F	24	++++	Deeply hemorrhagic at all sites with 1 dark blister	Edema of submucosa, infiltration of round cells, eosinophils, & polymorphonuclears in submucosa, muscularis & serosa; localized area of hemorrhage in mucosa
143	F	24	++++	Deeply hemorrhagic at all sites of terminal colon	Hemorrhage in muscularis; infiltration with eosinophils diffusely; collections in vessels; edema & cellular infiltration in submucosa
131	M	24	+++	3 of 5 sites had hyperemia & hemorrhage in terminal colon; 3 of 8 also had blisters in proximal	Proximal colon: edema, cellular infiltration, & hemorrhage in submucosa; dilated lymphatics, eosinophils Distal colon: edema & hemorrhage in submucosa; perivascular infiltrates, mucosal infiltrates & eosinophils
137	M	168	—	Negative	Minimal changes with increased cellularity in the lamina propria
142	F	168	—	Negative	Granulomatous involvement of serosa with giant cells (Fig. 17)

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Lang<sup>43</sup> considered tissue eosinophilia a "suspicious" sign of allergic reaction but by no means pathognomonic. Campbell et al.<sup>44</sup> regarded tissue eosinophilia as a definite accompaniment of allergy. Speirs and Dreisbach<sup>45</sup> observed a "tremendous" number of eosinophils in the peritoneal fluid after repeated injections of antigen. Speirs<sup>46</sup> later considered eosinophils as active cells in the early phases of antibody production against the antigen. Eosinophilia was noted in varying degree in the present material as diffuse or as large cellular conglomerates; eosinophiles are highly characteristic of the Arthus reaction.

*Swelling of Connective Tissue Fibers.*—Very little can be said about this feature in the present material. This was reported by Gerlach,<sup>17</sup> Opie,<sup>18</sup> Laporte,<sup>19</sup> Pagel,<sup>20</sup> Nicoletti,<sup>22</sup> and Berger and Lang,<sup>43</sup> who studied the skin. A possible explanation may be the difference between the chorion of the skin, rich in connective tissue fibers, and the intestine, which contains only very little loose connective tissue.

*Use of Adjuvants.*—No important difference was observed in relation to Schedule A or B; the changes in the tissues examined were comparable.

*Multiple-Site Reactions.*—There was no significant difference in reactivity when eliciting injections were made in multiple sites of the colon. The colon reacted vigorously to hyperimmune stimuli in all areas.

*Delayed Versus Anaphylactic Type of Response.*—It appears that leukocytes, seen more in the anaphylactic type of response, were present more conspicuously in the specimens obtained at 6-, 12-, and 24-hour intervals from the challenging injection. The later cellular responses were more of the histiocytic, perivascular, and monocytic types commonly associated with delayed hypersensitivity, confirming the observations of Dienes and Mallory,<sup>31</sup> who also noted a monocytic reaction replacing the leukocytic response in the 24-hour specimens when reactions induced with egg albumen were

examined. The tuberculin type of reaction was present in the first phases of allergy, according to these authors. Salvin,<sup>47</sup> using immunologic methods, found the delayed type of response to occur earlier in the experimental animal than the anaphylactic response. In these observations there was a dissociation of both responses, which could not be expected in the entirely different conditions of our experiment. Nevertheless, histiocytes, vascular proliferation, and granulomata, characteristic of tuberculin type of hypersensitivity, also are typical of the Arthus phenomenon.

*Control Group.*—The nonsensitized rabbits given albumen subserosally developed a moderate accumulation of leukocytes at the site of injection. One animal with a local accumulation of eosinophils was found at autopsy to have coccidiosis of the liver. The sensitized animals given saline as the challenging injection manifested frequent hemorrhages at the injection site, with a poor cellular response around these hemorrhagic foci. Granulomas were not observed in the control animals.

*Significance of Arthus Phenomenon for Human Disease.*—The Arthus reaction was important clinically when skin phenomena occurred as a result of administration of therapeutic antisera. The use of toxoids and antibiotics reduced their occurrence significantly. Could not certain diseases of the gastrointestinal tract be an expression of a locally occurring hypersensitive reaction? Kaiserling and Ochse<sup>48</sup> found all segments of the gastrointestinal tract responding to hyperimmune techniques in the experimental animal. The mucosa of the ileum, colon, and rectum in man is responsive to sensitization with reagins.<sup>49,50</sup> It seems improbable that hyperimmune phenomena do not occur spontaneously in the gastrointestinal tract. Morphologic criteria will not delineate completely the nature of such hyperimmune reactions should they occur; physiological, biochemical, and immunological techniques will be required to clarify this potentially important problem.

### Summary

Thirty white rabbits were sensitized to crystalline white albumen, and local Arthus reactions were induced in their colons through a transperitoneal approach. The colon responded to hyperimmune stimuli, and, on serial examinations over a period of four weeks, a sequence of events was observed, leading to histiocytosis, granuloma formation, and eosinophilia. The concept is suggested that in human disease where the local tissue manifests features encountered in the Arthus phenomenon hyperimmune mechanisms may be involved.

### Conclusion

The colon of rabbits responds to specific sensitization with a tissue reaction, characterized early by edema, hemorrhage, cellular infiltration, and damage of smooth muscle and later by granuloma and giant-cell formation and eosinophilia.

Department of Medicine, The University of Chicago (37).

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# The Architecture of the Conduction System in Congenital Heart Disease

## II. Tetralogy of Fallot

MAURICE LEV, M.D., Chicago

This is the second in a series of studies of the position and course of the conduction system in congenital heart disease. The first dealt with common atrioventricular (AV) orifice.<sup>1</sup> The present communication deals with tetralogy of Fallot. The historical aspect of this subject has been previously reviewed.<sup>1</sup>

### Materials and Methods

Four hearts, the seat of tetralogy of Fallot, were studied grossly and histopathologically. Specimen 4 was sectioned from the posterior walls of the atria and ventricles proceeding anteriorly. Specimens 1, 2, and 3 were sectioned in a manner previously described.<sup>2</sup> All sections were stained alternately with hematoxylin and eosin and Weigert-Van Gieson stains. The technical details are presented in the Table.

#### Technical Details

Specimen	Method of Sectioning	Thickness of Section, $\mu$	Slides, No.	Slides Lost, No.
1	q 20	8	306	0
2	q 20	7	290	0
3	q 20	8	224	0
4	Serial	10	1,880	7

#### Findings Specimen 1

##### Gross Examination (Fig. 1)

The heart was abnormal in shape. The apex was formed by both ventricles. From the base two arteries emerged, a larger

Submitted for publication Oct. 14, 1958.

This investigation was supported by a grant from the American Heart Association.

From the Congenital Heart Disease Research and Training Center, Hektoen Institute, and the Department of Pathology of the Northwestern University Medical School.

situated to the right, and a smaller to the left. The mutual relationships of the various chambers were normal.

The right atrium was larger than the left. It received the superior and inferior venae cavae and coronary sinus in a normal manner. The Eustachian valve was normal, but the Thebesian valve was absent. The limbus was well formed, and the foramen ovale was closed. The tricuspid orifice was large. The anterior and inferior leaflets of the tricuspid were connected to their usual papillary muscles. The medial leaflet was connected to a separate papillary muscle and to the muscle of Lancisi.

The right ventricle was large, and its wall almost equalled the thickness of the left ventricle. The architecture of the muscle bands of the right ventricle was abnormal. A powerful band ascended along the septum to the base of the heart, where it bifurcated into two parts. An inferior part turned to the right, forming the lower margin of the ventricular septal defect. The superior part ascended to the junction of the aorta and pulmonary artery. Here it formed an arch terminating in the upper part of the anterior wall of the right ventricle. The main inferior part of the band likewise formed an arch on the lower part of the anterior wall of the right ventricle. A separate muscle band extended from the base of the aorta to the anterior wall of the right ventricle in close proximity to the anterior leaflet of the tricuspid. Another muscle band bridged the left aspect of the cavity in a position reminiscent of the moderator band. It was not possible to say what corresponded to the normal septal and

## CONDUCTION SYSTEM—CONGENITAL HEART DISEASE

parietal bands of the crista. Emerging from this chamber were the large aorta and the small pulmonary artery. The aorta swung over the right bronchus and gave off the brachiocephalic vessels in a mirror-image position. The pulmonary artery emerged from a narrow conus. The endocardium in this region was white and thickened. The pulmonic valve consisted of three cusps. The pulmonary artery gave off its two branches in a normal manner. The ductus arteriosus was closed.

The left atrium was smaller than the right. It received the four pulmonary veins in a normal manner. The mitral orifice and valvular apparatus presented no change.

The left ventricular chamber was normal in size. The ventricular septum presented a defect in its anterior portion, measuring 1.0 cm. in greatest diameter. It was now apparent that the aorta straddled the interventricular septum, arising about one-third from the left and two-thirds from the right ventricle. The coronary arteries arose from the right and left posterior sinuses of Valsalva. The coronary circulation was abnormal. The artery arising from the left posterior sinus of Valsalva was small and gave off the ramus cristae and two collateral vessels. The artery arising from the right posterior sinus of Valsalva was large, giving off all the other branches. The coronary veins were not dissected.

### Anatomic Diagnosis

Corvisart's disease (tetralogy of Fallot with right aortic arch).

### Histologic Examination of Conduction System

The sino-atrial (SA) node lay in its normal position. The tricuspid and mitral annuli lay at about the same level and thus met the central fibrous body at about the same level (Fig. 2). The AV node originated from the lower distal part of the atrial septum on the right side, proximal and adjacent to the central fibrous body (Fig. 2). The AV bundle penetrated the central fibrous body as the conus musculature inserted on the central fibrous body. Thus the bundle was covered on its

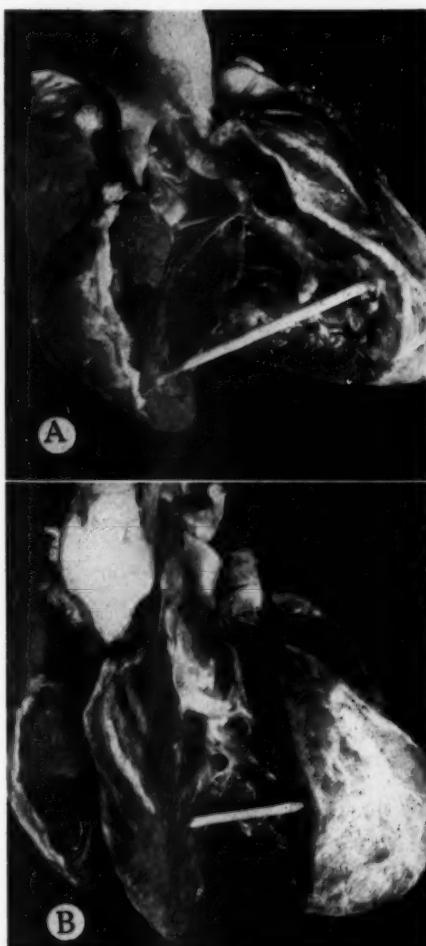
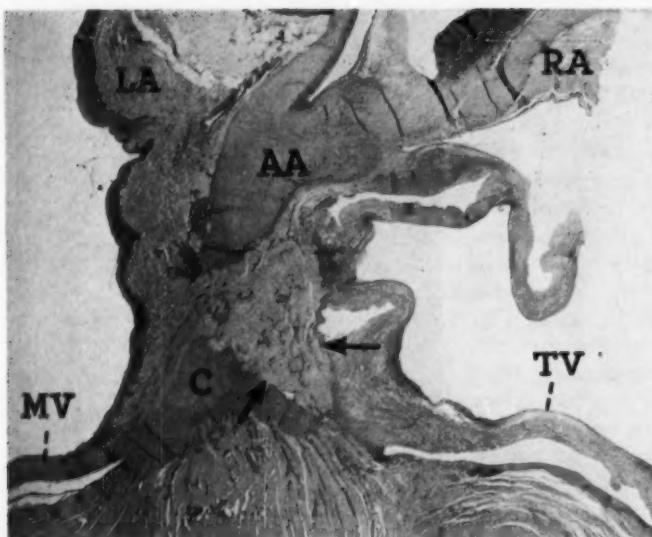


Fig. 1 (Specimen 1).—Right ventricular view of the heart. *A*, looking into the aorta. *B*, looking into the conus and the pulmonary artery.

right side by conus musculature and hence lay on the left side of the septum as it reached the level of the ventricular septal defect (Fig. 3). Here it flattened and widened out, lying on the left side of the septum below the defect (Fig. 4). It now gave off the posterior radiation of the left bundle branch. At the distal wall of the defect it bifurcated into the right bundle branch and the anterior radiation of the left. The anterior and posterior radiations of the left bundle branch were more compact



**Fig. 2 (Specimen 1).**—Oblique section through the AV node. Topographic view showing relationship to surrounding structures. *LA* indicates left atrial musculature; *RA*, right atrial musculature; *AA*, aortic annulus; *MV*, mitral valve; *TV*, tricuspid valve; *C*, central fibrous body; arrows point to the node. Weigert-Van Gieson stain;  $\times 8.5$ .

than normal, proceeding more clearly as cords to the papillary muscles (Fig. 5). The right bundle branch passed obliquely through the septum (Fig. 6) below the level of the defect to reach the right ventricular endocardium, beneath the conus

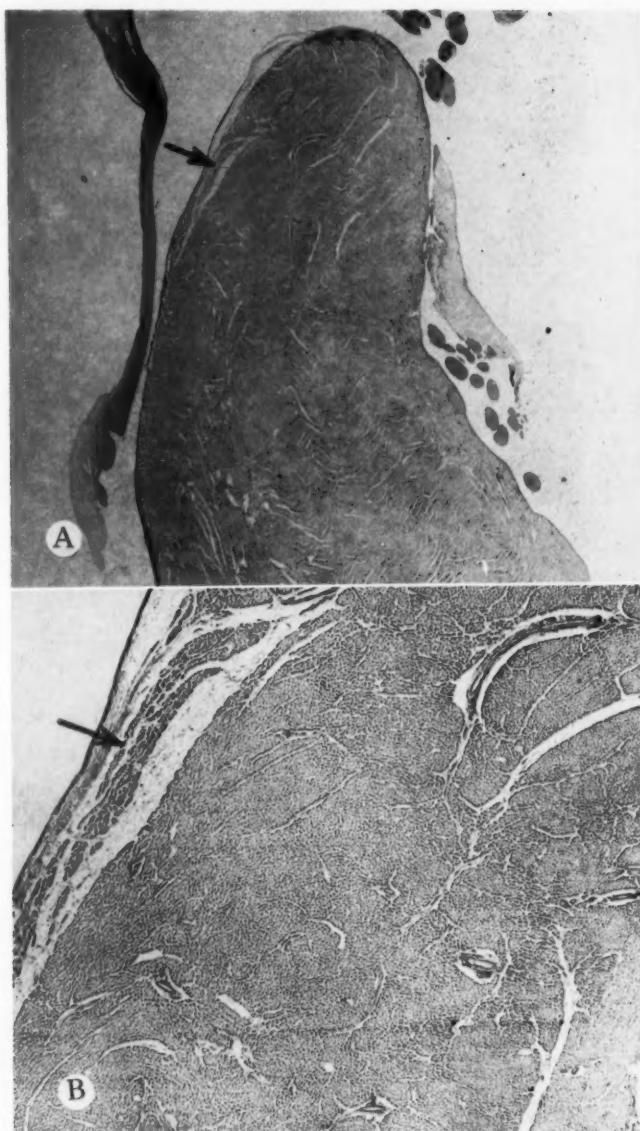
of the right ventricle. Here it lay adjacent to, but was not involved in, the area of fibroelastosis (Fig. 7). It then bifurcated into two parts. The larger part swung over one of the muscle bundles, bridging the right ventricular cavity described above, to fan



**Fig. 3 (Specimen 1).**—Section through the penetrating portion of the AV bundle. *V* indicates ventricular septal musculature; arrows point to the bundle. Hematoxylin and eosin;  $\times 34$ .

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Fig. 4 (Specimen 1).—Sections through the branching portion of the AV bundle. Arrows point to the bundle. *A*, Weigert-Van Gieson stain;  $\times 8.5$ . *B*, higher power of *A*;  $\times 34$ .



out over the parietal wall. The smaller part proceeded along the lower border of another muscle band toward the apex (Fig. 7).

**Specimen 2**

**Gross Examination (Fig. 8)**

The heart was globular in shape. The apex was formed by both ventricles. From

the base two arteries emerged, a larger situated to the right and slightly posterior and a smaller to the left and slightly anterior. The mutual relationships of the various chambers were normal.

The right atrium was moderately enlarged, and its wall was slightly hypertrophied. The superior and inferior venae



Fig. 5 (Specimen 1).—  
Section through the left  
bundle branch. Weigert-  
Van Gieson stain;  $\times 34$ .

cavae and coronary sinus entered this chamber normally. The mouth of the coronary sinus, however, was tremendous, as the latter received a large left superior vena cava. The Eustachian and Thebesian valves were represented by a common, markedly fenestrated curtain, which continued on the linea terminalis as a low

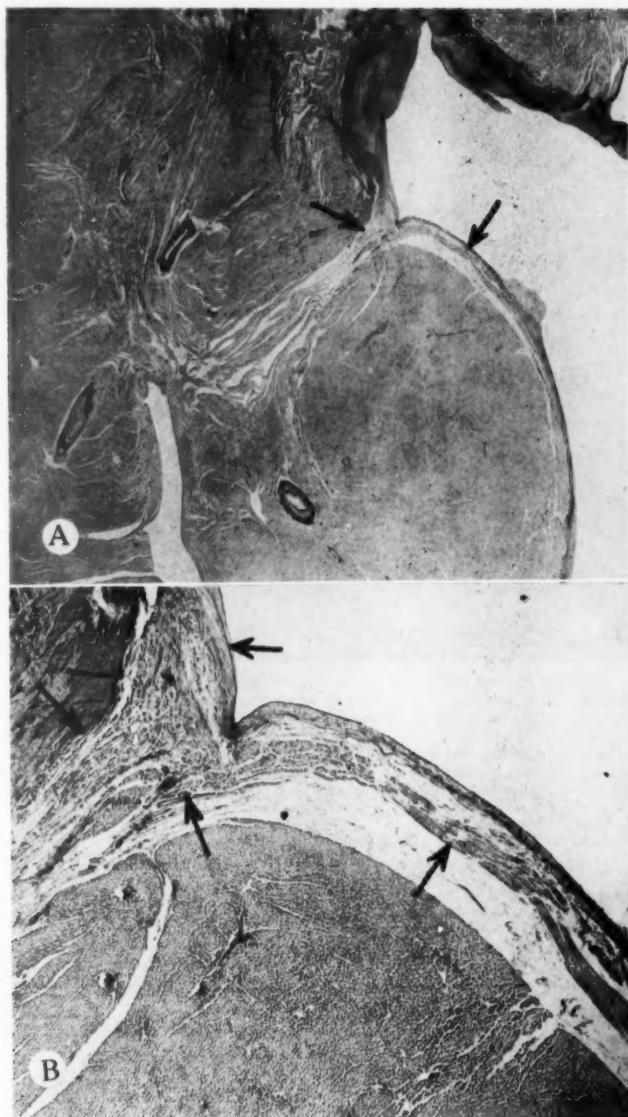
ridge and terminated at the entry of the right superior vena cava. The limbus was well formed but small. The septum primum was defective in the region of the limbus, producing a patent foramen ovale. The tricuspid valve consisted of two cusps, an anterolateral normal leaflet and a fused medial and inferior leaflet. The anterolateral



Fig. 6 (Specimen 1).—  
Section through right  
bundle branch as it passes  
through the septum in the  
distal wall of the defect.  
Arrow points to the right  
bundle branch. Weigert-  
Van Gieson stain;  $\times 34$ .

CONDUCTION SYSTEM—CONGENITAL HEART DISEASE

Fig. 7 (Specimen 1).—Sections through the right bundle branch as it reaches the subendocardium of the right ventricle, lying beneath the area of fibroelastosis. A, Weigert-Van Gieson stain;  $\times 8.5$ . B, higher power of A, showing the bifurcation of the right bundle branch;  $\times 34$ .



leaflet was connected to the anterolateral papillary muscle and the muscle of Lancisi in the normal manner. The fused medial and inferior leaflets were connected to the muscle of Lancisi and the inferior papillary muscle.

The right ventricular cavity was enlarged, and its wall was thickened, it being almost

as thick as that of the left ventricle. The architecture of the muscle bundles of the right ventricle was abnormal. The septal muscle bundle was represented by two separate muscle bands. The inferior of these commenced at the anterolateral papillary muscle and proceeded upward on the septum to terminate at the left margin of

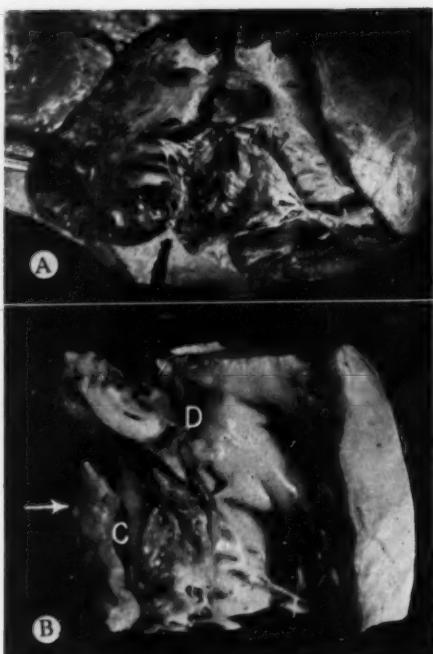


Fig. 8. (Specimen 2).—*A*, right ventricular view looking into the ventricular septal defect and the conus. *B*, the block as taken for section of the AV node, bundle, and branches. Right atrial and right ventricular view. *C* indicates entry of coronary sinus; *D*, ventricular septal defect; arrow points to the direction of sectioning.

the interventricular foramen. The superior one ran roughly parallel to this and terminated at the base of the pulmonic valve and the left superior margin of the defect. The parietal muscle bundle likewise was represented by two bundles. The superior terminated at the base of the aorta and the inferior at the medial portion of the anterior tricuspid leaflet. Only a fine muscle ridge bridged the gap between the superior parietal and the superior septal muscle band. Two arteries emanated from this chamber. The larger of these was the aorta, which arose in the hiatus between the septal and parietal muscle bands. The smaller of these was the pulmonary artery, which emerged from its usual position. The pulmonic valve consisted of two cusps. The pulmonary artery divided in a normal manner into right and left branches. The left branch

was markedly dilated at its distal end where it was anastomosed with the aorta. The ductus arteriosus was closed.

The left atrium was small in comparison to the right. It received the four pulmonary veins in a normal manner. The mitral orifice was small but normal in configuration. The mitral valve, chordae tendineae, and papillary muscles presented no change.

The left ventricle was smaller than the right. Its wall was slightly thicker than the right. The anterior portion of the ventricular septum presented a defect measuring about 1.0 cm. in diameter. Over this defect straddled the large aorta. The aortic valve and coronary ostia and distribution were normal. The coronary veins were not dissected. There were four brachiocephalic vessels which were given off in the following order: (1) the right common carotid, (2) the left common carotid, (3) the left subclavian, and (4) the right subclavian. Thus, the right subclavian arose from the descending aorta. A well-healed surgical anastomosis was present between the thoracic aorta and the left pulmonary artery.

#### Anatomic Diagnosis

1. Tetralogy of Fallot
  - (a) Bicuspid pulmonic valve
  - (b) Patent foramen ovale
2. Left superior vena cava entering the coronary sinus
3. Right subclavian from the descending aorta
4. Common Eustachian and Thebesian valves
5. Potts procedure

#### Histologic Examination of Conduction System

The SA node was not available for study. The musculature of the distal inferior atrial septal wall which normally lies distal to the entry of the coronary sinus was deviated in a horizontal plane by the distal wall of the markedly enlarged coronary sinus. The beginning of the AV node which develops in this musculature was likewise deviated and was seen to be draped over the central fibrous body, lying more to the left of and above this body than normally (Fig. 9).

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Fig. 9 (Specimen 2).—Section through the AV node, draped over the central fibrous body. *C* indicates central fibrous body; arrows point to the AV node. Weigert-Van Gieson stain;  $\times 34$ .



More distally, it assumed its normal position to the right of the central fibrous body and adjacent to the tricuspid valve. The tricuspid annulus lay on a more proximal level than the mitral annulus. The bundle of His penetrated the central fibrous body as the anterior ventricular septal musculature joined the latter, covering the distal part of the right side of the penetrating

portion of the bundle. Thus, the AV bundle in its further course lay on the left side of the summit of the ventricular septum at the level of and below the ventricular septal defect. It now proceeded to give off the left posterior radiation. The branching bundle now flattened out. At the level of the distal wall of the defect it gave off the right bundle branch (Fig. 10). The

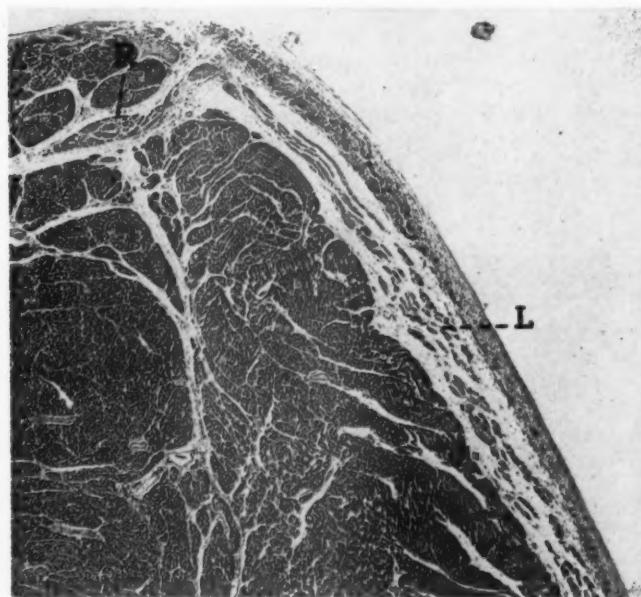


Fig. 10 (Specimen 2).—Sections through the bifurcation of the AV bundle into the right bundle branch and the anterior radiation of the left. *R* indicates right bundle branch; *L*, anterior radiation of the left bundle branch. Hematoxylin and eosin;  $\times 34$ .

latter penetrated the musculature and moved obliquely through the septum to reach its right subendocardial surface. Here it lay in the lower aspect of the septal band, and it proceeded to the moderator band. After the origin of the right bundle branch, the left bundle branch continued to give off numerous fasciculi which constituted the anterior radiation. However, the anterior was not easily delineated from the posterior radiation. Thus, the left bundle branch consisted of a more compact mass than normal, which only distally presented a sharper delineation into anterior and posterior radiations.

### Specimen 3

#### *Gross Examination (Fig. 11)*

The heart was typically boot-shaped. The apex was formed by both ventricles. From the base two arteries emerged, a larger situated to the right and a smaller situated to the left. The mutual relationships of the various chambers were normal.

The right atrium was somewhat larger than the left. It received the superior and inferior venae cavae and coronary sinus in a normal manner. The Eustachian valve was normal. The Thebesian valve was absent. The limbus was well formed, and the foramen ovale was closed. The tricuspid orifice was normal in size. The anterior

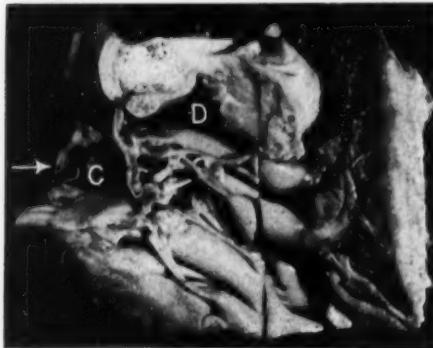
tricuspid leaflet was connected to the anterolateral papillary muscle and to a markedly hypertrophied muscle of Lancisi. The medial leaflet was connected to numerous small papillary muscles. The inferior leaflet was connected to a small inferior papillary muscle.

The right ventricle was markedly hypertrophied. The septal muscle bundle was represented by two hypertrophied bands. The thicker of the two was short and lay superiorly beneath the pulmonic valve. It was continuous with the thick parietal muscle bundle passing over the anterior wall of the right ventricle, forming the crista. The inferior septal band passed to the inferior margin of the defect (Fig. 11). Another parietal band lay beneath the aortic valve and adjacent to the tricuspid valve. It fused with the other parietal band to form one common parietal muscle bundle. From the right ventricle emerged the aorta and pulmonary artery. The conus of the right ventricle was narrowed and led into the pulmonary artery. The pulmonary orifice was small, and its valve consisted of two cusps. The pulmonary artery divided into its two branches normally. The ductus arteriosus was closed.

The left atrium was small. It received the four pulmonary veins in a normal manner. The mitral orifice and valvular apparatus presented no change.

The left ventricle was of equal thickness to the right. Its chamber, however, was small. The ventricular septum presented a defect measuring 1.0 cm. in greatest diameter. This defect was in the posterior part of the anterior ventricular septum. From this chamber emerged the large aorta. It was now evident that the aorta emerged from both ventricles. The aortic valve consisted of three cusps. The left coronary emerged from the left posterior sinus of Valsalva, while the right emerged from the anterior. The coronary distribution was normal. The coronary veins were not dissected. The innominate and the left common carotid emerged from a common trunk. The left subclavian was normal.

Fig. 11 (Specimen 3).—Right atrial and ventricular view of block taken for section of the AV node, bundle, and bundle branches. C indicates entry of coronary sinus; D, ventricular septal defect; arrow points to the direction of cutting.



## CONDUCTION SYSTEM—CONGENITAL HEART DISEASE

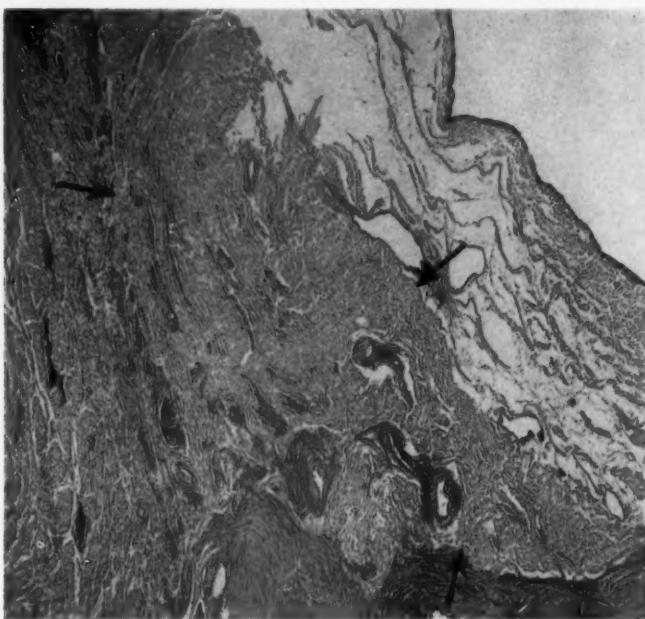


Fig. 12 (Specimen 3).—  
Oblique section through  
the AV node. Arrows point to the node. Weigert-Van Gieson stain;  
 $\times 34$ .

### Anatomic Diagnosis

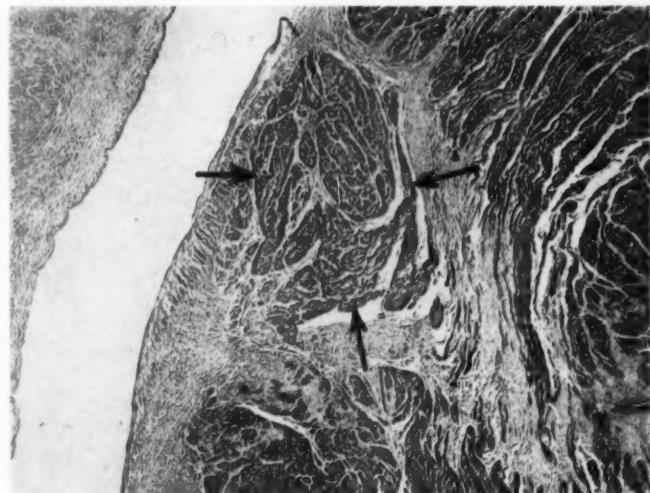
Tetralogy of Fallot.

### Histologic Examination of Conduction System

The SA node was not available for study. The annuli of the mitral and tricuspid valves were attached to the central fibrous body at about the same level. The AV node

originated in the right side of the inferodistal portion of the atrial septum, adjacent to and proximal to the central fibrous body (Fig. 12). It penetrated the central fibrous body to become the AV bundle as the aorta came down to meet the central fibrous body. At this point the musculature of the anterior ventricular

Fig. 13 (Specimen 3).—  
Section through the penetratin portion of the AV bundle lying on the left side of the septum. Arrows point to the bundle. Hematoxylin and eosin;  
 $\times 34$ .



septum was attached to the central fibrous body, and so the bundle was covered on its right side by conus musculature and lay on the left side of the septum, as it reached the posterior margin of the ventricular septal defect (Fig. 13). It now flattened out, giving off the fasciculi of the anterior radiation, as it coursed below the defect on the left side of the summit of the ventricular septum. It then bifurcated at the level of the distal part of the defect into the right bundle branch and the anterior radiation of the left. The right bundle branch migrated through the septum obliquely to reach the endocardium of the right ventricle beneath the overhanging parietal band. Here it divided into two parts, which were distributed to both septal bands. The anterior and posterior radiations of the left bundle branch were more compact than normal.

#### Specimen 4

##### *Gross Examination*

The heart was minute. The apex was formed by both ventricles. From the base two arteries emerged, a larger one situated posteriorly and to the right and a smaller anteriorly and to the left. The mutual relationships of the various chambers were normal.

The right atrium was of average size and thickness. The superior and inferior venae cavae and coronary sinus entered this chamber in a normal manner. The Eustachian valve was large and extended upward on the parietal wall on to the posterior crest. The Thebesian valve was normal. The limbus was well formed but made a wide arc. The foramen ovale was probe patent in two areas. The septum primum was somewhat redundant, producing an aneurysm of the fossa ovalis. The tricuspid orifice was normal in size. The tricuspid valve consisted of three leaflets. The medial and inferior leaflets were both connected to a very large posteroseptal papillary muscle. The anterolateral leaflet was small but normally connected to the anterior papillary muscle.

The right ventricle was hypertrophied, its wall just about equalling that of the left. The architecture of the muscle bands of the right ventricle was abnormal. The septal muscle band was represented by two separate muscle bands. The inferior of these proceeded up the septum to terminate on the inferior margin of the ventricular septal defect. The superior of these proceeded up to the septum to the base of the pulmonic valve, where it joined the superior of two parietal bands to form the crista supraventricularis. Beneath this parietal band there was another muscle band which lay in close proximity to the tricuspid. This terminated at the base of the aorta. From the right ventricle two arteries emerged. The larger was the aorta, which originated in the hiatus between the inferior parietal and inferior septal bands. The smaller was the pulmonary trunk, which originated from the conus in the usual manner. The pulmonic valve was bicuspid. The pulmonary trunk gave off the two pulmonary arteries in a normal manner. The ductus arteriosus was patent with a small diameter. The left atrium was smaller than the right. The mitral valve, chordae tendineae, and papillary muscles showed no change.

The left ventricle was normal in size, and its wall equalled that of the right ventricle. The anterior portion of the ventricular septum presented a large defect at its base, measuring 0.5 cm. in diameter. The remainder of the ventricular septum was normal. The aorta emerged from the two ventricles in a straddling position, two-thirds from the left ventricle and one-third from the right. The right coronary cusp was situated anteriorly; the left, posteriorly and to the left, and the posterior, posteriorly and to the right. The left coronary ostium arose above the sinus of Valsalva. The brachiocephalic arteries were given off normally. The right coronary artery formed the ramus obtusi, while the left circumflex terminated in the anterior wall of the left ventricle. The coronary veins were not dissected.

CONDUCTION SYSTEM—CONGENITAL HEART DISEASE

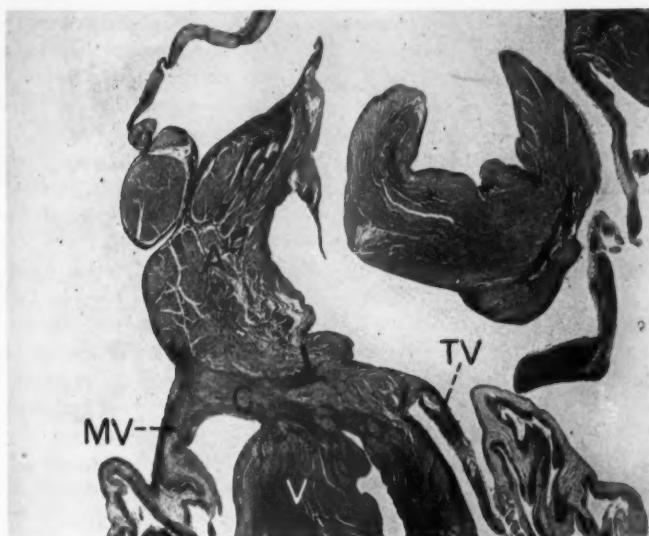


Fig. 14 (Specimen 4).—Horizontal view of the beginning of the penetrating portion of the AV bundle. *MV* indicates mitral valve; *TV*, tricuspid valve; *C*, central fibrous body; *A*, atrial septum; *V*, ventricular septum; arrow points to the penetrating portion of the bundle. Weigert-Van Gieson stain;  $\times 13$ .

Anatomic Diagnosis

1. Tetralogy of Fallot
- (a) Bicuspid pulmonic valve

- (b) Large Eustachian valve
- (c) Aneurysm of the fossa ovalis
- (d) Patent ductus arteriosus



Fig. 15 (Specimen 4).—Horizontal view of the distal portion of the AV bundle, lying in the floor of the ventricular septal defect on the left side of the ventricular septum, and fasciculi of the left bundle branch. *B* indicates bundle; *M*, remnant of pars membranacea; *U*, muscular ventricular septum; *L*, fasciculi of left bundle branch. Hematoxylin and eosin;  $\times 50$ .

### *Histologic Examination of Conduction System*

The SA node was normal. The AV node lay in the inferodistal part of the atrial septal wall, on the right side between the central fibrous body and the medial leaflet of the tricuspid. The tricuspid and mitral annuli were about on the same level. The node invaded the central fibrous body, thus becoming the AV bundle (Fig. 14). It then proceeded beneath the remnant of the pars membranacea interventricularis, where it gave off numerous Mahaim fibers to the ventricular septum. It then began giving off the posterior radiation of the left bundle branch. It reached the level of the defect as conal musculature appeared on the right side, and so the bundle lay on the left side of the summit, below the defect (Fig. 15). At the distal wall of the defect the bundle divided into the right bundle branch and the left anterior radiation. The right bundle branch passed obliquely through the septum to become subendocardial about halfway between the apex and base and reached the moderator band. There was no sharp line of demarcation between the anterior and the posterior radiations of the left bundle branch.

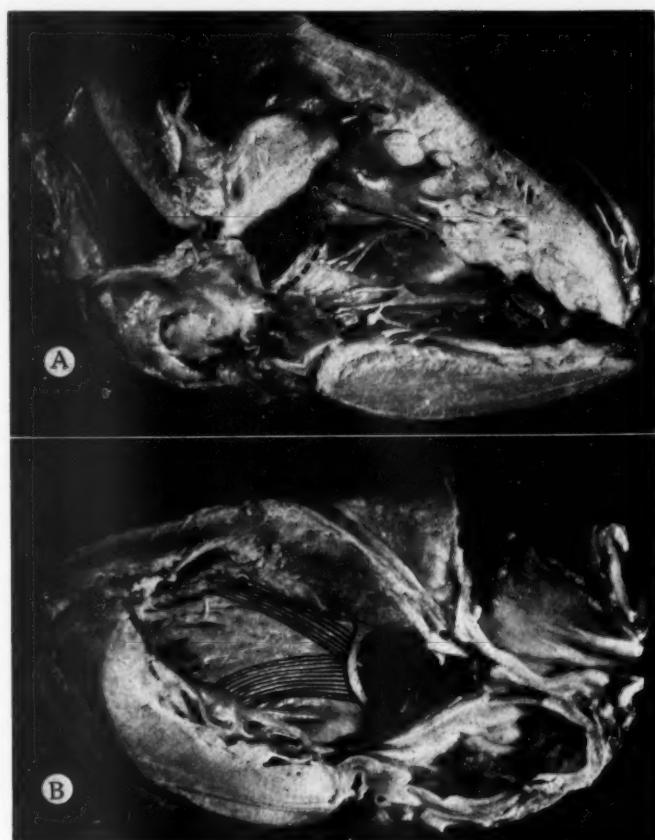
### **Comment**

Tetralogy of Fallot, as is well known,<sup>3-6</sup> is a type of overriding aorta complex, where the latter is associated with pulmonary tract narrowing, ventricular septal defect, and therefore of necessity right ventricular hypertrophy. The defect is situated in the posterior part of the anterior (bulbar) septum and involves most or all of the pars membranacea. The left atrium and left ventricle are in general smaller than normal. The medial leaflet of the tricuspid may be attached by small papillary muscles to the septum or may be fused with the anterior or the inferior leaflet (mitralization of the tricuspid). The architecture of the muscle bundles of the right ventricle is abnormal. Most often the single or doubled septal band is hypertrophied, with its upper component fusing with a displaced

parietal band to form the abnormal crista supraventricularis. The parietal band is deviated away from the tricuspid valve over the anterior wall of the right ventricle, thus becoming a component in the narrowing of the infundibular tract. In mild overriding the crista lies above the defect. In marked overriding the crista lies distal to and above the defect. The coronary ostia may be rotated in a counterclockwise direction, looking ventriclesward from the aorta. The annulus of the aorta is displaced to the right and meets the central fibrous body more to the right than normal. The annuli of the tricuspid and mitral valves are more or less on the same level and meet the central fibrous body at about the same level, in contrast to the normal, where the tricuspid annulus lies more distally.

The course of the conduction system in the uncomplicated case of tetralogy of Fallot is as follows (Fig. 16A and B): The SA node is in the normal position. The AV node arises in the inferodistal aspect of the atrial septum (Fig. 2). This part of the septum is abbreviated, owing to the position of the tricuspid and mitral annuli. The AV node penetrates the central fibrous body as normally to become the AV bundle (Figs. 3 and 14). The bundle may give off Mahaim fibers as normally. If there is an upward extension of the central fibrous body (pars membranacea), then as normally the bundle may lie at the base of this portion of the ventricular septum. The bundle then proceeds to the left side of the septum as the conal musculature of the right ventricle joins the central fibrous body and as the level of the defect is reached (Fig. 4). However, the bundle which lies on the most anterior part of the posterior septum is not directly related to the defect, since the posterior boundary of the defect is lined by conal musculature. Thus the bundle lies on the left side of the ventricular septum (as is true of some normal hearts), just below the defect (Figs. 4, 13, and 15). The bundle then begins to give off fasciculi of the left bundle branch. At the distal wall of the defect it divides into the right bundle branch

Fig. 16.—Diagrammatic sketch of the course of the conduction system in tetralogy of Fallot. Atrial and ventricular septa of a specimen of tetralogy of Fallot. The parietal walls of the heart have been removed to better view the ventricular septal defect. *A*, right ventricular view. *B*, left ventricular view. In *A* dotted line represents AV node; dash line, the bundle of His present on the left side of the septum; dot-dash line, the right bundle branch within the myocardium, and full line, the right bundle branch subendocardially. In *B*, the dense line represents the bundle of His and the beginning right bundle branch. The fine lines represent the anterior and posterior radiations of the left bundle branch.



and remainder of the left (Fig. 10). The right bundle branch passes through the septum to reach the right subendocardial part of the septum (Figs. 6, 7, and 10). Here the normal architecture of the right bundle branch may be changed by the altered architecture of the muscle bundles. Thus there may be two right bundle branches (Fig. 7B). If there is fibroelastosis of the conus, the right bundle branch may lie immediately adjacent to this area (Fig. 7A) and perhaps in some cases may be involved in this process. The left bundle branch has a tendency to be more compact and in some cases not readily divisible at its origin into anterior and posterior radiations (Fig. 5). When the tetralogy is complicated by a left superior vena cava entering

the coronary sinus, then the AV node may be deviated in a horizontal plane (Fig. 9).

Thus, the architecture of the conduction system is altered in the following way in tetralogy of Fallot: 1. The AV bundle lies on the left side of the ventricular septum below the defect. 2. The right bundle branch may be divided into two (or perhaps more) parts. 3. The left bundle branch may be more compact and less divisible into anterior and posterior radiations till further down in the septum. 4. If there is fibroelastosis of the conus, the right bundle branch lies adjacent to it. 5. If there is a left superior vena cava entering the coronary sinus, the AV node is deviated horizontally.

The mild alterations present are understandable in the light of the embryogenesis

of the conduction system and of the possible embryogenesis of tetralogy of Fallot.

The AV node and bundle originate from the atrial canal musculature. They originate from the musculature of the posterior part of this canal, which lies behind the posterior endocardial cushion at a time when the musculature of this canal is still unbroken.<sup>7-12</sup> The primordia of these structures appear before the inferior septum has joined the posterior endocardial cushion and before the endocardial cushions have fused. There is a difference of opinion as to whether both the AV node and bundle originate *in situ* or whether the bundle originates from a proliferation of AV nodal tissue. There is also a difference of opinion as to whether the bundle branches originate *in situ* from the ventricular trabeculae or whether they originate from a proliferation of the tissue of the bundle. In man, the AV node and bundle originate at about 8.0-10.0 mm., and at 13.0 mm. the left bundle branch has developed considerably and the right first makes its appearance. At 16.5 mm. the left bundle branch is completely developed, and at about 22.0-25.0 mm. the right bundle branch is traced to the moderator band. Histologic differentiation of these structures is a continuing process up to birth.

The embryogenesis of tetralogy of Fallot has intrigued embryologists, anatomists, and pathologists for many years.<sup>13-23</sup> The various theories which have been advanced as to the embryogenesis were reviewed by Lev and Saphir,<sup>18,19</sup> Harris and Farber,<sup>24</sup> and, more recently, by Vossenaar.<sup>22</sup> The more modern theories consider tetralogy of Fallot to be related to an abnormality in the development and incorporation of the bulbus or bulbotruncal area into the ventricles. Various theories consider the cause of such abnormal incorporation to lie in an abnormality in the bulbar ridges or bulbar septum, an abnormality in the bulboauricular spur or crest or conoventricular flange, an abnormality in the right aspect of the anterior endocardial cushion, or any combination of these. On the other hand, the

embryogenesis of the AV node and bundle are related to the posterior endocardial cushion and the posterior septum (septum ventriculorum proprium). Hence, it could be anticipated that the course of the conduction system would not be altered greatly in tetralogy of Fallot. Owing to the aortic annulus meeting the central fibrous body more to the right than normal, the mitral and tricuspid annuli lie on the same level in contrast to the normal, where the mitral lies more proximal than the tricuspid. Thus the AV node still lies adjacent to the tricuspid valve and the central fibrous body, but it is the latter two structures which have somewhat altered their position. Of course, where a left superior vena cava enters the coronary sinus then the distal wall of the ostium of the coronary sinus, which normally forms the proximal boundary of the AV node, of necessity now compresses and rotates the AV node in a horizontal plane, and so its proximal portion may lie draped on the central fibrous body, but more distally it occupies its usual position adjacent to the annulus of the tricuspid valve. The AV bundle lies below and basically not directly related to the ventricular septal defect, because the latter is in the anterior (bulbar) septum in contrast to the AV bundle in isolated ventricular septal defect, which lies on the upper border of the posterior ventricular septum. Since the right bundle branch originates from the trabecular structure of the right ventricle or is elaborated on the trabecular structure of the right ventricle, where the latter as in tetralogy of Fallot is abnormally formed, then it presents an altered architecture. Likewise, the left bundle branch similarly elaborated is now more compact, owing to the altered architecture of the trabeculae of the left aspect of the ventricular septum due to hemodynamic alterations related to the overriding aorta and the ventricular septal defect. Where there is fibroelastosis of the conal area, then the right bundle branch, which normally borders but lies below the conus, will lie adjacent to the fibroelastosis, and it may be anticipated that

## CONDUCTION SYSTEM—CONGENITAL HEART DISEASE

in some cases it will be involved in the fibroelastosis.

### Summary

The course of the atrioventricular (AV) node, bundle, and bundle branches in four cases of tetralogy of Fallot is described.

There are mild variations from the normal related to the abnormal position of the aorta and the abnormal topography of the right ventricle.

The position of the AV node may be altered by a left superior vena cava entering the coronary sinus.

The AV bundle is situated on the left side of the summit of the ventricular septum, below the ventricular septal defect.

The altered embryogenesis of the AV node, bundle, and bundle branches in tetralogy of Fallot is discussed.

Congenital Heart Disease Research and Training Center, Hektoen Institute for Medical Research, 629 S. Wood St. (12).

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## News and Comment

### PERSONAL

**Dr. Wiley D. Forbus Delivers Lecture.**—Dr. Wiley D. Forbus delivered the Caldwell Lecture at the University of Texas Southwestern Medical School on Nov. 30, 1958. He talked on "The Emergence of Mycotic Infectious Disease and Its Impact on Medicine."

**Medical Woman of the Year.**—Dr. Eleanor M. Humphreys has been named Medical Woman of the Year (1958) by the American Medical Women's Association.

### GENERAL NEWS

**Forensic Pathology Registry.**—A Registry of Forensic Pathology has been established at the Armed Forces Institute of Pathology, Washington, D. C. It will be the 24th component of the American Registry of Pathology, and it is under the sponsorship of the College of American Pathologists. The objective of the new registry is the collection of well-checked and well-documented medicolegal cases to be used as a reservoir for teaching and research. Only selected cases of medicolegal interest contributed by a qualified pathologist will be registered. The material will be incorporated in the files of the Armed Forces Institute of Pathology and become a part of its national collection of pathology.

Registry consultation on contributed cases will be limited to basic pathological changes and will not include medicolegal opinions which might involve Armed Forces Institute of Pathology staff members in court proceedings. Cases are now being accepted for registration. A six-month fellowship in forensic pathology at the Armed Forces Institute of Pathology has been provided by the college as a part of its sponsoring support.

### ANNOUNCEMENTS

**Histochemistry Course.**—A laboratory course in histochemistry will be offered June 8 to 19, 1959. The first week will be concerned with techniques of tissue preparation, including freeze-drying, freeze-substitution, and carbowax embedding, and the second week, with histochemical techniques for enzymes and metallic ions. Tuition will be \$75 per week. Guest faculty will include Drs. E. Farber, of New Orleans; F. B. Johnson, of Washington; A. G. E. Pearse, of London, and H. Yokayama, of Chicago.



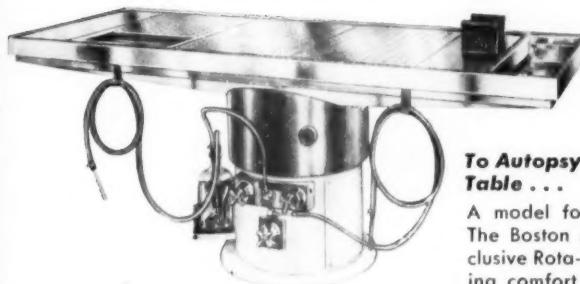
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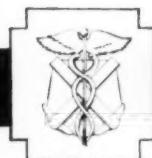
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